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29

16. Juni 2005

Notice of Opposition to a European Patent

To the
European Patent Office

Tabulation Marks

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I. Patent opposed Zur Kasse A. € 610,-

Opp. No. OPPO (1)

Patent No. 0 656 786

Application No. 93909679.8-2107

Date of mention of the grant in the European Patent Bulletin (Art. 97(4), 99(1) EPC) 15.09.2004

Title of the invention: "Use of Isoflavone Phyto-Oestrogen Extracts of Soy or Clover"

II. first named in the patent specification

Novogen Research Pty Ltd

Proprietor of the Patent

Opponent's or representative's reference (max. 15 spaces) 136.05 OREF

III. Opponent OPPO (2)

Name: Strawman Limited

Address: 34 Lovedon Lane, Winchester, Hampshire, SO23 7NU, UK

State of residence or of principle place of business: GB

Telephone/Telex/Fax:

Multiple opponents: ☐ further opponents see additional sheet

IV. Authorisation

1. Representative OPPO (9)

(Name only one representative to whom notification is to be made)

Name: SHEARD, Andrew Gregory

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Additional representative(s): ☒ ROBERTS, Alison Christine OPPO (5)

2. Employee(s) of the opponent authorised for these opposition proceedings under act. 133(3) EPC: Name(s):

Authorisation(s) To 1./2.:

☒ not considered necessary

☐ has/have been registered under No.

☐ is/are enclosed

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V. Opposition is filed against

— the patent as a whole ☒

— claim(s) No(s).

VI. Grounds for opposition:

Opposition is based on the following grounds:

(a) the subject-matter of the European patent opposed is not patentable (Art. 100(a) EPC) because:

— it is not new (Art. 52(1); 54 EPC) ☒

— it does not involve an inventive step (Art.52(1); 56 EPC) ☒

— patentability is excluded on other grounds, i.e. Art. ☐

(b) the patent opposed does not disclose the invention in a manner sufficiently clear and complete for it to be carried out by a person skilled in the art (Art. 100(b) EPC; see Art. 83 EPC). ☒

(c) the subject-matter of the patent opposed extends beyond the content of the application/ of the earlier application as filed (Art. 100(c) EPC, see Art. 123(2) EPC). ☒

VII. Facts and arguments

(Rule 55(c) EPC)
presented in support of the opposition are submitted herewith on a separate sheet ("Opposition Statement") ☒

VIII. Other requests:

It is requested that the Patent be revoked in its entirety.

Oral proceedings are requested if the Patent cannot be revoked on the basis of the written submissions alone. If oral proceedings take place and the Patentee or any other party chooses to speak in French or German, simultaneous translation into English is requested.

IX. Evidence presented

for EPO use only

Enclosed = ☐

will be filed at a later date = ☒

A. **Publications:** | Publication date

D1 Messina M. and Barnes S., "The Role of Soy Products in Reducing Risk of Cancer" *Journal of the National Cancer Institute* **83**(8) 541-546 (1991) — 1991

Particular relevance (page, column, line, fig.): *See Opposition Statement*

D2 Beckham N, "The Family Guide to Natural Therapies", 1988, page 41 — 1988

Particular relevance (page, column, line, fig.): *See Opposition Statement*

D3 EP-A-0135172 (Takeda) — 27-03-1985

Particular relevance (page, column, line, fig.): *See Opposition Statement*

D4 WO-A-9423716 (Tufts University) — 27-10-1994 15-04-1994 (Art 54(3))

Particular relevance (page, column, line, fig.): *See Opposition Statement*

D4P USS/N 08/049,006 — 16-04-1993 (Art 54(3))

Particular relevance (page, column, line, fig.): *See Opposition Statement*

D5 Wilcox *et al.*, *Br. Med. J.* **301** 905-906 (1990) — 20-10-1990

Particular relevance (page, column, line, fig.): *See Opposition Statement*

D6 JP-A-01258669 (Kikkoman) — 16-10-1989

Particular relevance (page, column, line, fig.): *See Opposition Statement*

Continued on additional sheet ☒ (see page 5)

B. **Other evidence**

Continued on additional sheet ☐

X. Payment of the opposition fee is made

for EPO use only

☐ as indicated in the enclosed voucher for payment of fees and costs (EPO Form 1010)

☒ Payment has already been made online, as evidenced by the enclosed copy debit order. In case of any irregularity with that payment, the EPO is authorised to debit the correct amount from my deposit account 2805 0305.

XI. List of documents:

Enclosure No.:			No. of copies	
0	☒	Form for notice of opposition	2 (min. 2)	
1	☒	Facts and arguments ("Opposition Statement") (see VII.)	2 (min. 2)	
2		Copies of documents presented as evidence (see IX.)		
2a	☐	— Publications	(min. 2 of each)	
2b	☐	— Other documents	(min.2 of each)	
3	☐	Signed authorisation(s) (see IV.)		
4	☐	Voucher of payment of fees and costs (see X.)		
5	☐	Cheque		
6	☒	Additional sheet (*page 5 of this form*)	2 (min. 2 of each)	
7	☒	Other (please specify here):	Copy debit order	

XII. Signature of opponent or representative

Place Berkhamsted, Herts., UK

Date 14 June 2005

A. G. Sheard, Authorised Representative

Please print name under signature. In the case of legal persons, the position which the person signing holds within the company should also be printed.

IX. Evidence presented – Continued Enclosed = ☐ will be filed at a later date = ☒	for EPO use only
B. Publications:	Publication date
D6A English translation of **D6** Particular relevance (page, column, line, fig.): *See Opposition Statement*	n/a
D7 JP-A-61246124 (Yamanouchi) Particular relevance (page, column, line, fig.): *See Opposition Statement*	01-11-1986
D7A English translation of **D7** Particular relevance (page, column, line, fig.): *See Opposition Statement*	n/a
D8 Adlercreutz *et al.*, *The Lancet* **339** 1233 (1992) Particular relevance (page, column, line, fig.): *See Opposition Statement*	16-05-1992
D9 Declaration of Graham Edmund Kelly filed before the ED, with exhibits GK1 and GK2 Particular relevance (page, column, line, fig.): *See Opposition Statement*	n/a

EUROPEAN PATENT NO: **EP-B-0656786**
Application No: 93909679.8-2107

"Use of Isoflavone Phyto-oestrogen Extracts of Soy or Clover "

PATENTEE: Novogen Research Pty Ltd

OPPONENT: Strawman Limited

OPPOSITION STATEMENT

1 INTRODUCTION

(1) This constitutes Opponent Strawman Limited's statement under Rule 55(c) EPC, namely the statement of the extent to which European Patent No. EP-B-0656786 ("the Patent") is opposed and of the grounds on which the opposition is based as well as an indication of the facts, evidence and arguments presented in support of these grounds.

(2) The following documents are introduced into the proceedings, with assigned document numbers. In the event that there is more than one opposition, it is suggested that the Patentee compile a master document list.

Doc No.	Patentee's Number	Document	Published
D1		Messina M. and Barnes S., "The Role of Soy Products in Reducing Risk of Cancer" *Journal of the National Cancer Institute* **83**(8) 541-546 (1991)	1991
D2		Beckham N, "The Family Guide to Natural Therapies", 1988, page 41	1988
D3		EP-A-0135172 (Takeda)	27-03-1985
D4		WO-A-9423716 (Tufts University)	27-10-1994 15-04-1994 (Art 54(3))
D4P		USS/N 08/049,006	16-04-1993 (Art 54(3))
D5		Wilcox *et al.*, *Br. Med. J.* **301** 905-906 (1990)	20-10-1990

Doc No.	Patentee's Number	Document	Published
D6		JP-A-01258669 (Kikkoman)	16-10-1989
D6A		English translation of D6	
D7		JP-A-61246124 (Yamanouchi)	01-11-1986
D7A		English translation of D7	
D8		Adlercreutz *et al.*, *The Lancet* **339** 1233 (1992)	16-05-1992
D9		Declaration of Graham Edmund Kelly filed before the ED, with exhibits GK1 and GK2	n/a

(3) All the claims of the Patent are opposed.

(4) The grounds on which the Patent is opposed are:

- Art 100(c)/Art 123(2) EPC – Added subject-matter
- Art 100(b)/Art 83 EPC – Insufficiency
- Art 100(a)/Art 54 EPC – Lack of novelty
- Art 100(a)/Art 56 EPC – Lack of inventive step

2 THE PATENT

2.1 Formalities

(5) The Patent was granted on European Patent Application No. 93909679.8, which was derived from PCT Patent Application No. PCT/AU93/00230 ("the PCT Application" or "the Application as filed"), which was filed on 19 May 1993 ("the Filing Date") and published as WO-A-9323069. Priority was claimed from Australian Provisional Patent Application No. PL2511/92 ("the Priority Application") filed on 14 June 1995 ("the Priority Date").

2.2 The Claims

(6) All the claims are in second medical use format and relate to various medical uses of an isoflavone phyto-oestrogen extract of soy or clover. Claim 1 is the sole independent claim. Claims 2 to 11 are all dependent, directly or indirectly, on claim 1.

3 REQUESTS

(7) It is requested that the Patent be revoked in its entirety.

(8) Oral proceedings are requested if the Patent cannot be revoked on the basis of the written submissions alone. If oral proceedings take place and the Patentee or any other party chooses to speak in French or German, simultaneous translation into English is requested.

4 ADDED SUBJECT-MATTER

4.1 "...an isoflavone phyto-oestrogen extract of soy or clover"

(9) Subject matter has been added by the reference in claim 1 as granted to "an isoflavone phyto-oestrogen extract of soy or clover". In the Application as filed, the disclosure was limited to the use of genistein, daidzein, biochanin A, and/or formononetin. There was no disclosure in the Application as filed that the invention could be practiced without any of the four specified compounds, as claim 1 as granted now contemplates. In his letter of 18 August 1999 before the Examining Division (ED) the then Applicant referred to page 8, lines 8 to 16, page 10, lines 12 and 13 and pages 11 to 13 of the Application as filed in support of wording broader than the four specified compounds. But all this discussion in the Application as filed is exclusively in the context of the four specific isoflavones to which the invention is stated to relate:

> DISCLOSURE OF INVENTION
> The present invention concerns a health supplement specifically enriched for isoflavones selected from genistein, daidzein, formononetin and biochanin A, or their natural glycoside form, or their analogues, in sufficient amounts to improve the health of a human. [Application as filed, page 8, lines 4-7]

Read in context, therefore, the passages relied upon do not establish basis for any isoflavones other than the four specified. The fact that genistein, daidzein, formononetin and biochanin A may be the *principal* isoflavones of soy and clover, as argued by the Applicant at the foot of page 2 of his letter of 18 August 1999, is irrelevant since there is no suggestion that they are the *only* isoflavones of those plants.

4.2 "...in unit dosage form"

(10) Subject-matter was also added to claim 1 by the introduction of the words "in unit dosage form". Although certain unit dosage forms were disclosed in the

Application as filed (claim 7, which was dependent on claim 1), those unit dosage forms obligatorily contain particular phyto-oestrogens (namely genistein, daidzein, biochanin A and/or formononetin, as specified in claim 1 as filed) an amount of from about 20 mg to 200 mg. In the Patent, the skilled person is presented with new information, namely that the unit dose form may contain (a) *any* isoflavone phyto-oestrogen extract of soy or clover and (b) phyto-oestrogen in an amount *other than* from about 20 mg to 200 mg.

4.3 "...or derivatives thereof"

(11) Subject matter has been added by the inclusion in claim 6 of the phrase "or derivatives thereof" relating to the two phyto-oestrogens specified in the claim. The Application as filed discloses on page 1, second paragraph only "*closely related* derivatives" of phyto-oestrogens in general.

5 INSUFFICIENCY

5.1 Enabling Disclosure for Second Medical Use Claims

(12) All the claims of the Patent are second medical use claims. The Boards have held that to provide an enabling disclosure for such claims it is essential for an application or patent to provide evidence that the alleged medicament in fact has the required biological activity (**T497/02**, **T609/02**); a mere vague indication of a possible medical use is not enough. In the present case, the Patent contains no evidence that the phyto-oestrogen extract recited in claim 1 of the Patent has any activity in the treatment of pre-menstrual syndrome, symptoms associated with menopause or prostate cancer.

(13) The fact that evidence not in the Patent may support the medical uses referred to in the claims does not help the Patentee. As the Board noted in **T609/02**:

> 9. Where a therapeutic application is claimed in the form allowed by the Enlarged Board of Appeal in its decision G5/83 (OJ EPO 1985, 64), ie in the form of the use of a substance or composition for the manufacture of a medicament for a defined therapeutic application, attaining the claimed therapeutic effect is a functional technical feature of the claim (see G 2/88 and G 6/88, OJ EPO 1993, 93 and 114, Headnote III. and point 9 of the reasons, for non-medical applications, see also T 158/96 of 28 October 1998, point 3.1 of the reasons). As a consequence, under Article 83 EPC, unless this is already known to the skilled person at the priority date, the application must disclose the suitability of the product to be manufactured for the claimed

> therapeutic application. ... **It is required that the patent provides some information in the form of, for example, experimental tests, to the avail that the claimed compound has a direct effect on a metabolic mechanism specifically involved in the disease, this mechanism being either known from the prior art or demonstrated in the patent per se.** [T609/92 ¶9, emphasis added]

5.2 Single Phyto-Oestrogens

(14) Claim 1 covers the use of a single phyto-oestrogen. This is confirmed by claim 6, which specifies that the extract comprises "one or more of" the compounds specified. There is no disclosure in the Patent how to prepare an extract containing only one phyto-oestrogen.

5.3 Ratio of Phyto-Oestrogens

(15) Likewise, there is no disclosure in the Patent how to prepare an isoflavone phyto-oestrogen preparation containing the ratio specified in claim 7.

6 PRIORITY

(16) Before the questions of novelty and inventive step can properly be addressed, the priority entitlement of the claimed subject matter has to be established.

(17) Since the Priority Application does not contain any claims, any priority basis for the claimed subject matter needs to be present in the description of the Priority Application. According to **G2/98**, priority can be acknowledged for a given claim only if the skilled person can derive the subject-matter of the claim directly and unambiguously, using common general knowledge, from the Priority Application as a whole.

6.1 Claim 1

(18) There are at least three reasons why claim 1 is not entitled to priority.

(19) First, claim 1 is not entitled to the Priority Date because the following features cannot be directly and unambiguously derived from the Priority Application. The absence of either one of these features from the Priority Application is enough to deprive the claim of priority entitlement:

a. "extract of soy or *clover*": the Priority Document deals almost exclusively with soy. In the context of the disclosed invention, only one particular species of clover, namely subterranean clover, is disclosed as an alternative source of isoflavones (page 9, third complete paragraph):

> An alternative source of these isoflavones is subterranean clover (Trifolium spp.), many varieties of which have isoflavone levels of the order of 5% of dry weight. Compared to soya, however subterranean clovers are less advantageous because of the relative absence of coumestans.

b. "a medicament for administration in *unit dosage form*": the expression "unit dosage form" does not appear in the Priority Application.

(20) Secondly, the Priority Application does not specifically disclose the claimed *combination* of (a) the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer with (b) an isoflavone phyto-oestrogen. The three different medical conditions recited in the claim are selected from a list on page 11; and so-called type 4 phyto-oestrogens – isoflavones – are selected from a list of different phyto-oestrogens on pages 1 and 2 of the Priority Application. This particular combination of selections is not disclosed in the Priority Application.

(21) Thirdly, it is a well established principle of law that a priority document must contain an enabling disclosure for it to provide a valid priority basis (**T193/95** *etc.*; see "Case Law of the Boards of Appeal of the EPO", Fourth Edition, pages 242-244). Furthermore, in the particular case of second medical use claims, the Boards have held that to provide an enabling disclosure it is essential for an application to provide evidence that the alleged medicament in fact has the required biological activity (**T497/02**, **T609/02**). The same logic applies to priority entitlement under Art 87 EPC as to sufficiency under Art 83 EPC. In the present case, the Priority Application contains no evidence that the phyto-oestrogen extract recited in claim 1 of the Patent has any activity in the treatment of pre-menstrual syndrome, symptoms associated with menopause or prostate cancer.

(22) Consequently, claim 1 is not entitled to the benefit of the Priority Date, and both it and every other claim dependent on it must be assessed for novelty and inventive step as of the Filing Date.

6.2 Claim 5

(23) Claim 5 specifies that the phyto-oestrogen is extracted from clover. As noted above in relation to claim 1, the Priority Document discloses that isoflavones can only be extracted from one particular species of clover, namely subterranean clover (page 9, third complete paragraph). Claim 5 is therefore not entitled to priority.

6.3 Claim 7

(24) Claim 7 is not entitled to priority, since neither the specified range of ratios nor the two phyto-oestrogens biochanin A and formononetin appear in the Priority Document.

6.4 Claim 8

(25) Claim 8 is not entitled to priority, since the specified weight ranges are not disclosed in the Priority Document.

6.5 Claim 9

(26) Claim 9 is not entitled to priority, since administration of a medicament as defined in claim 1 at least daily over a period of at least a month is not disclosed in the Priority Document.

6.6 Claim 10

(27) Claim 10 is not entitled to priority, since an extract the extract including coumestans, lignans and flavones is not disclosed in the Priority Document. Page 12, second complete paragraph, merely discloses isoflavones and/or coumestans.

7 LACK OF NOVELTY

7.1 D1 (Messina & Barnes)

(28) **D1** is citable under Art 54(2) EPC, irrespective of the priority entitlement of the claims of the Patent.

(29) **D1**, which includes a section aptly entitled "Isoflavones in Cancer Prevention" (page 541), includes a report (page 542, paragraph 5) on a study undertaken on the effects of feeding soy to postmenopausal women, by the provision of *nutritional supplements*, whereby subjects consumed one main soy dish (1/2 cup of soybeans or 38g texturised vegetable protein) and two soy snacks, either soy

chips (a roasted soybean product) or a spread for crackers, made from the whole soybean. The supplements were taken daily over a four week period and the estimated isoflavone content was about 200mg/day. It was reported, that, compared to control subjects, significantly more women that were fed soy exhibited an oestrogenic response, as demonstrated by an increase in the number of superficial cells of the vaginal epithelium. The treatment of symptoms associated with the menopause is thus directly and unambiguously derivable from **D1**.

(30) **D1** also discloses the treatment of these symptoms with an *extract* of soy, as required by claim 1. The term "extract" is not defined in the Patent, but we see from Example 5, that soy hypocotyls are administered to humans. A hypocotyl, which is a part of the soy plant, is therefore regarded by the Patentee as an "extract", otherwise Example 5 could not be an example of the invention. If a soy hypocotyl is an "extract", then so is a soy bean, as used in **D1**.

(31) Other soy "extracts" disclosed in **D1** which are not apparently excluded from the scope of claim 1 include: texturised vegetable protein prepared from soy; soy chips; and "a spread for crackers, made from the whole soybean".

(32) **D1** therefore deprives claim 1 of novelty.

7.2 D2 (Beckham)

(33) **D2** is citable under Art 54(2) EPC, irrespective of the priority entitlement of the claims of the Patent.

(34) **D2** discloses the use of red clover and soy (soya) beans to treat symptoms of the menopause (page 41) and pre-menstrual syndrome (page 50). Soy beans are extracts of soy (see Section 7.1 above). **D2** discloses the features of claim 1.

7.3 D3 (EP-A-0135172)

(35) **D3** is citable under Art 54(2) EPC, irrespective of the priority entitlement of the claims of the Patent.

(36) **D3** discloses certain isoflavones (including 7,4'-dihydroxyisoflavone or daidzein) in the treatment of osteoporosis – see claim 1 of **D3**. These isoflavone

phyto-oestrogens are disclosed as having oestrogenic activity (page 3, line 11). Osteoporosis is associated with menopause (page 1, lines 14-15).

(37) Claim 1 specifies the use of "an isoflavone phyto-oestrogen extract of soy or clover". But this wording affords no distinction from **D3**, because it merely specifies how the isoflavone phyto-oestrogen has been prepared. It has long been part of EPO law and practice that a claim to a product when prepared by a particular process has to be interpreted as a claim to the product *per se*, independently of the process (**T219/83**, **T248/85**). This principle applies not only to products which are themselves the subject of a claim, but also to products which are referred to in process or use claims (**T620/99**). Therefore claim 1 reads onto the use of an isoflavone phyto-oestrogen whether or not it has, as a matter of historical fact, been prepared by extraction from soy or clover, or by synthetic organic chemistry or in any other way. Accordingly, claim 1 lacks novelty over **D3**.

7.4 D4 (WO-A-9423716)

(38) **D4** was referred to before the ED as D15. Insofar as it is supported by the contents of **D4P**, **D4** is prior art by virtue of Art 54(3) EPC, since the claims of the Patent are not entitled to the Priority Date, as explained in Section 5.2 above. This explanation does not seem to have been considered by the ED.

(39) **D4** and **D4P** disclose the features of claim 1. For example:

> The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. [**D4**, page 1, lines 24-28; **D4P**, page 1, lines 22-26]
>
> 1. Use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen [**D4**, claim 1]
>
> 1. A method of preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen, said method comprising administering to the woman an effective amount of an isoflavonoid. [**D4P**, claim 1]

(40) Claim 1 accordingly lacks novelty over **D4** as supported by **D4P**.

7.5 D5 (Wilcox et al.*)*

(41) **D5** is citable under Art 54(2) EPC, irrespective of the priority entitlement of the claims of the Patent.

(42) **D5** discloses the administration to post-menopausal women (page 905, last paragraph) a diet supplemented with soya flour (45 g daily) and red clover sprouts (10 g dry seed daily) (page 906, lines 1-2). The severity of the menopause was modulated (page 906: data, left column; conclusion, right column).

(43) Claim 1 accordingly lacks novelty over **D5**.

8 LACK OF INVENTIVE STEP

8.1 Problem/Solution Analysis

(44) According to the case law formulated by the Boards of Appeal and set out in the Guidelines for Examination in the European Patent Office ("the Guidelines") at C:IV 9.5, the analysis of the question of inventive step should be conducted by the three-step problem/solution analysis:

- determine the closest prior art;
- establish the technical problem to be solved; and
- consider whether or not the claimed invention, starting from the closest prior art and the technical problem, would have been obvious to the skilled person.

(45) Implicit in the problem/solution analysis is the requirement that the problem must actually be solved by the claimed invention.

8.2 D3 as the Closest Prior Art

(46) As noted above, **D3** (EP-A-0135172) discloses certain isoflavones (including 7,4'-dihydroxyisoflavone or daidzein) in the treatment of osteoporosis – see claim 1 of **D3**. The isoflavones are disclosed as having oestrogenic activity (page 3, line 11). Osteoporosis is associated with menopause (page 1, lines 14-15).

(47) We have shown under Section 7.3 above that claim 1 lacks novelty over **D3**. But even if the Patentee is able to establish that the isoflavone phyto-oestrogen

specified in the claim is somehow different from that disclosed in **D3**, claim 1 lacks an inventive step. The problem to be solved is merely to provide an alternative source of isoflavones for the same purpose. The solution to this problem claimed by the Patent is to use an isoflavone phyto-oestrogen extract of soy or clover.

(48) This solution is obvious because **D6** teaches that daidzein and other isoflavones can be isolated from soybeans (see **D6A** page 1, section 2 and Examples 1-4). Claim 1 consequently does not involve an inventive step.

8.3 D7 as the Closest Prior Art

(49) **D7** was referred to before the ED as D2. **D7** discloses that genistein is isolated from clover (**D7A**, page 2, fourth paragraph) and has carcinostatic action, as determined by its ability to inhibit (a) proliferation of RSV-transformed rat cells (RSV-3Y1 cells), human epithelial cancer cells (A431 cells) and SV40-transformed rat cells (SV40-3Y1 cells) and (b) DNA synthesis in mouse mastocytoma (P815) cells and mouse thymus (EL-4) cells.

(50) The problem to be solved is the treatment of prostate cancer. The solution to that problem proposed by the Patent is to use, among other things, genistein extracted from clover.

(51) That solution is obvious. It is nothing more than the treatment proposed in **D7**. Before the ED, the Applicant filed on 21 October 2003 a declaration by the inventor Dr Kelly (**D9**), who argued (¶¶6-9) that a person skilled in the art would not be led by the teaching of **D7** to the claimed invention since genistein was not a pan-cancer treatment. If this is correct, though, it has profound consequences for the sufficiency of the disclosure of the Patent, which has *no* data relating to the efficacy of *any* of the compounds used in claim 1 in the treatment of *any* cancer, prostate or otherwise.

8.4 D8 (Adlercreutz et al.*)*

(52) **D8** was considered by the ED, especially in the Examination Report dated 11 August 2003. Before the ED, **D8** was known as D14. **D8** discloses that:

a. hot flushes and other menopausal symptoms are infrequent in Japanese women (**D8**, first and last sentences);

b. the lower incidence of hot flushes in Japanese women compared to Canadian women cannot be explained by "cultural indifference" (**D8**, first paragraph);

c. high levels of isoflavonoid phyto-oestrogens were found in the urine of (i) a group of women whose mean age was 50.4 (SD 18.0) from a small village south of Kyoto and (ii) a group of three men, three women and three children living in Kyoto (**D8**, middle paragraph);

d. the excretion of the isoflavonoids was associated with intake of soy products (**D8**, last paragraph); and

e. the high levels of the isoflavonoid phyto-oestrogens may explain the infrequency of hot flushes and other menopausal symptoms in Japanese women (**D8**, last sentence).

(53) On the basis of **D8**, the problem to be solved is to provide a treatment for hot flushes and other symptoms associated with the menopause. The solution envisaged by claim 1 is to provide *inter alia* an isoflavone phyto-oestrogen extract of soy. That solution is obvious. It logically flows from the observations in **D8**.

(54) The applicant's response to the citation of **D8** was to file declarations by the inventor (**D9** and **D9-GK1**) and a Dr Claude Hughes (**D9-GK2**). The inventor described **D8** as "a speculative reference, which is indefinite, uncertain and tentative" (**D9** ¶13). However, the fact is that **D8** contains far more evidence than the Patent does about the likely effectiveness of an isoflavone phyto-oestrogen extract of soy in the treatment of symptoms associated with the menopause.

(55) Likewise, in **D9-GK2**, Dr Hughes has various criticisms about the data in **D8**. In ¶17 he says that **D8** does not report on the menopausal symptoms of the women enrolled in the study[1]. And in ¶18 he expresses a concern about lack of controls. Interestingly, however, Dr Hughes expresses no opinion on the quality of

[1] This criticism somewhat misses the point. **D8**'s observations on menopausal symptoms concern Japanese women as a whole, not those involved in the study.

the data in the Patent, which not only fails to report on menopausal symptoms but also is devoid of relevant data, let alone controls[2].

(56) Given the standards of data in the Patent that the Patentee considers to be acceptable, the criticisms of the data in **D8** are trifling. Claim 1 lacks an inventive step over **D8**.

8.5 The Patent does Not Disclose that any Problem is Solved

(57) The discussion of **D7** and **D8** above, and the applicant's comments in relation to these documents during prosecution, lead on to a more general point. In the present case, the supposed solution to whatever problem is established on the basis of the prior art is the use specified in claim 1, namely :

> The use of an isoflavone phyto-oestrogen extract of soy or clover, for the manufacture of a medicament for administration in unit dosage form for the treatment of pre-menstrual syndrome, symptoms associated with menopause, or prostate cancer.

But the Patent is completely devoid of any examples of the treatment of either (i) pre-menstrual syndrome, or (ii) symptoms associated with menopause, or (iii) prostate cancer.

(58) Examples 3 and 4 purport to show a fall in cholesterol levels, but that is not one of the medical indications specified in the claim[3]. Example 4 contains an anecdotal report of amelioration of a patient's benign breast disease problem and a further anecdotal report of regularisation of a patient's menstrual cycle and reduced menstrual bleeding. Even if these reports were to be significant (and there is no evidence that they are), they are not examples of any of the medical indications specified in the claim.

(59) The Patent therefore does not disclose that any problem has actually been solved. This places the Patentee in a somewhat awkward position. He is caught in a fork between insufficiency on the one hand and lack of inventive step on the other hand. Either the skilled person could practise what is claimed in claim 1 already on

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[2] "And why beholdest thou the mote that is in thy brother's eye, but considerest not the beam that is in thine own eye?" [Matthew VII verse 3]

[3] And Example 3 is said to be "not of the invention" in any event.

the basis of the prior art, or both the prior art and the Patent contain insufficient information to realise what is claimed in claim 1. This was exactly the position confronting the patentee in **T794/94**, which related to an unexemplified process for directly expressing mammalian proteins:

> 3.4.4. In view of the above finding, the board is unable to formulate any problem in relation to claim 1, for which it can be said that it has been solved by the novel information. It can also not be said that the actual contribution to the state of the art made by the disclosure of the patent in suit consists of providing experimental support for the direct expression of mammalian proteins, i.e., the technical contribution is not a new technique but the successful completion of an experiment known at a theoretical level from the prior art, as in the case dealt with in decision T 694/92 (OJ EPO 1997, 408). This is because the direct expression of mammalian proteins is not exemplified in the patent in suit. Therefore, either the skilled person could make what is claimed in claim 1 already on the basis of the prior art, or both the prior art and the patent in suit contain insufficient information to realize what is claimed in claim 1. Thus claim 1 must fail either as the requirements of Article 83 EPC have not been fulfilled, or for lack of inventive step (Article 56 EPC). In the absence of clear evidence that the equivalent information provided by the prior art, or by the patent in suit, is insufficient to allow the skilled person to carry out the invention, the board finds that claim 1 lacks an inventive step.

(60) Likewise, in the present case, claim 1 must fail either as the requirements of Article 83 EPC have not been fulfilled, or for lack of inventive step (Article 56 EPC).

9 CONCLUSIONS

(61) Subject-matter was added to the Application during prosecution. The Patent does not meet the required standard for sufficient disclosure applicable to second medical use claims. The claims define subject mater which is not new and which does not involve an inventive step. The Patent should be revoked in its entirety.

A. G. Sheard
Authorised Representative

14 June 2005

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14 June 2005

Your ref.: 0656786/93909679.8-2107
Our ref.: 136.05

Dear Sirs,

European Patent No. EP-B-0656786/93909679.8-2107
Novogen Research Pty Ltd (assignee of KELLY, Graham Edmund)
Opposition by Strawman Limited

On behalf of Strawman Limited of 34 Lovedon Lane, Winchester, Hampshire, SO23 7NU, UK, I hereby file

OPPOSITION

against the above patent. Revocation of the Patent in its entirety is requested. Further details are given on the enclosed EPO Form 2300 and accompanying statement under Rule 55(c) EPC. The Opposition fee has been paid online.

Please stamp and return the enclosed EPO Form 1037 as evidence of safe receipt.

Yours faithfully,

A. G. Sheard

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15 June 2005

Your ref.: 0656786/93909679.8-2107
Our ref.: 136.05

Dear Sirs,

European Patent No. EP-B-0656786/93909679.8-2107
Novogen Research Pty Ltd (assignee of KELLY, Graham Edmund)
Opposition by Strawman Limited

I file herewith a copy of each of the following documents:

Doc No.	Document
D1	Messina M. and Barnes S., "The Role of Soy Products in Reducing Risk of Cancer" *Journal of the National Cancer Institute* **83**(8) 541-546 (1991)
D2	Beckham N, "The Family Guide to Natural Therapies", 1988, page 41
D3	EP-A-0135172 (Takeda)
D4	WO-A-9423716 (Tufts University)
D4P	USS/N 08/049,006
D5	Wilcox *et al.*, *Br. Med. J.* **301** 905-906 (1990)
D6	JP-A-01258669 (Kikkoman)
D6A	English translation of D6
D7	JP-A-61246124 (Yamanouchi)
D7A	English translation of D7
D8	Adlercreutz *et al.*, *The Lancet* **339** 1233 (1992)
D9	Declaration of Graham Edmund Kelly filed before the ED, with exhibits GK1 and GK2

Only one copy of each of the above documents is filed herewith, as I understand that in view of the current practice of scanning all received documents the EPO no longer insists on – or indeed wants – strict compliance with Rule 59 EPC. However, I shall willingly file further copies if invited to do so in accordance with Rule 59.

Please stamp and return the enclosed EPO Form 1037 as evidence of safe receipt of this letter.

Andrew G. Sheard MA (Cantab.) LLB (London)
Chartered Patent Attorney European Patent Attorney

ANDREW SHEARD

Yours faithfully,

A. G. Sheard

A. G. Sheard
Authorised Representative

COMMENTARY

The Role of Soy Products in Reducing Risk of Cancer[1]

Mark Messina, Stephen Barnes*

Since the initial recognition that diet plays a role in the etiology of certain cancers, particularly cancers of the breast and colon, considerable progress has been made in identifying dietary patterns associated with cancer risk. There is general agreement that a high-fat, low-fiber diet, like that consumed by much of the industrialized world, increases cancer risk and that plant-based diets, rich in whole grains, legumes, and fruits and vegetables, are protective. It has been, however, considerably more difficult to identify specific foods, types of food, or components of foods that influence cancer risk.

The recent workshop on The Role of Soy Products in Cancer Prevention, sponsored by the National Cancer Institute, had two objectives: 1) to evaluate the role of soybeans, food products derived from soybeans, and specific components of soybeans in the dietary prevention of cancer and 2) to recommend research initiatives and approaches for further studies of the effect of soy intake on human cancer risk. The meeting was chaired by Stephen Barnes and organized by Mark Messina.

Isoflavones in Cancer Prevention

Kenneth Setchell, Donna Baird, and Barnes discussed the potential role of isoflavones in the prevention of cancer. Setchell reviewed the history of phytoestrogens (*1*), noting that plants were first observed to induce estrus in animals in 1926. Over 300 plants are now known to possess estrogenic activity (*2,3*). In 1946, the infertility observed in Australian sheep that grazed on a certain type of subterranean clover was attributed to the high isoflavone content of this plant (*4*). Ruminal bacteria in these animals convert plant isoflavones into the mammalian isoflavone equol, which, following absorption, may suppress the pituitary gonadotropic axis. Equol, a weak estrogen possessing about 0.2% of the biological activity of estradiol, was first identified in human urine in 1982 by Setchell et al (*5,6*). Setchell's further interest in the potent estrogenic effects of soybean isoflavones was stimulated coincidentally. He discovered that the soy component of diets fed to captive cheetahs, which was added for economic reasons, was responsible for the severe breeding problems in these animals (*6,7*).

Setchell noted that isoflavone metabolism has been studied in humans, although only superficially. In one study, subjects fed 40 g of soy daily were found to have urinary levels of equol as much as 1000-fold higher than baseline values (*8,9*). The low levels of urinary equol in two of the six subjects in this study indicate that the intestinal microflora (*10*) participate in isoflavone metabolism and that isoflavones undergo enterohepatic circulation (*10*). Improved analytical methods (*11,12*) have led to the realization that equol represents only a small fraction of the total amount of isoflavone in urine and that conjugates of the soybean isoflavones daidzein and genistein are the major forms present. The high levels of isoflavone in urine in subjects fed soy suggest that these compounds are likely to elicit a biological response (*13*).

Setchell concluded his presentation with a reminder (*a*) that all weak estrogens can also have antiestrogenic activity; (*b*) that tamoxifen, which has been used therapeutically for breast cancer, is structurally related to some of the phytoestrogens; and (*c*) that vegetarians, who may have a lower risk of certain cancers, excrete higher levels of phytoestrogens. These findings have led to collaborative studies by Barnes, Setchell, and associates (*14*), who used an animal model designed to test the hypothesis that phytoestrogens have a role in reduction of breast cancer risk.

[1]Report of a workshop held June 26-27, 1990, at the Guest Quarters Hotel in Bethesda, Md. Workshop members were Donna Baird, National Institute of Environmental Health Sciences, Research Triangle Park, NC; Stephen Barnes, University of Alabama at Birmingham, Birmingham, Ala; David L. Brandon, Western Regional Research Center, United States Department of Agriculture, Albany, Calif; James A. Duke, Agricultural Research Service, United States Department of Agriculture, Beltsville, Md; Ernst Graf, The Pillsbury Co, Minneapolis, Minn; Ann R. Kennedy, University of Pennsylvania Medical School, Philadelphia; Renee M. Kosslak, Iowa State University, Ames; Irvin E. Liener, University of Minnesota, St. Paul; Mark Messina, National Cancer Institute, Bethesda, Md; Frank L. Meyskens, University of California, Irvine, Calif; A. Venket Rao, University of Toronto, Ontario, Canada; Kenneth D. R. Setchell, Children's Hospital, Cincinnati, Ohio; Bernie F. Szuhaj, Central Soya, Fort Wayne, Ind.

Received October 22, 1990; revised January 8, 1991; accepted January 16, 1991.

S. Barnes, University of Alabama at Birmingham, Birmingham, Ala.
**Correspondence to:* Mark Messina, Diet and Cancer Branch, Division of Cancer Prevention and Control, National Cancer Institute, Bethesda, MD 20892.

Barnes began by observing that Oriental women, who have low incidence rates of breast cancer (*15*), consume larger amounts of soy products than do most American women. However, although fertility and reproduction in animals are adversely affected by ingestion of plant isoflavones, the amount of isoflavones in soy products consumed by Oriental women does not appear to affect their reproductive capacity.

Barnes discussed the recent animal study conducted in collaboration with Setchell and other investigators (*14*). In that study, consumption of soybeans significantly decreased chemically induced rodent mammary cancer. Rats were fed one of four soy products: powdered soybean chips consisting of unpurified soybeans, both raw and autoclaved; soy protein isolate composed of 91% protein; soy molasses, a concentrate of the aqueous alcohol extract of soy flour; and aqueous alcohol-extracted soy protein concentrate. All diets were isocaloric and isonitrogenous and produced similar weight gain among the animal groups throughout the study.

The first three products, all of which are rich in isoflavones, inhibited mammary tumorigenesis induced by 7,12-dimethylbenz[*a*]anthracene or methylnitrosourea, while the aqueous alcohol-extracted soy protein concentrate, which had a low content of isoflavones, did not. Whether the soybeans were raw or cooked made no difference in the degree of inhibition of mammary cancer; cooked soybeans were shown to be devoid of protease inhibitor activity.

Barnes said the reduction in levels of mammary tumor estrogen receptors induced by the powdered soybean chips paralleled the inhibition of tumorigenesis and supported the hypothesis that the isoflavones exerted an antiestrogenic effect. Interestingly, however, this was not the case for the soy protein isolate. The decrease in levels of mammary tumor estrogen receptors was smaller than predicted from the degree of tumor inhibition, he said, suggesting that the antiestrogenic effect of isoflavones may not be the primary mechanism responsible for inhibition of tumorigenesis. Therefore, Barnes concluded, the anticarcinogenic activity of isoflavones may not be limited to tumors containing a functional steroid receptor system. Alternative mechanisms of action may include inhibition of the activity of tyrosine protein kinases (eg, epidermal growth factor receptor tyrosine kinase) (*16*), DNA topoisomerase II (*17*), and ribosomal S6 kinase (*18*), as well as induction of specific cytochrome P450s (*19*).

Baird, before describing her recent study of the effects of feeding soy to postmenopausal women (manuscript in preparation), cited the concern of the National Institute of Environmental Health Sciences about the possible effects of low-level environmental estrogens on health. In her study, changes in estrogenic activity in postmenopausal women consuming soy over a 4-week period were examined. Soy was chosen for this study because of its high estrogenic activity (*20,21*), its increasing use in the United States, and the variety of products derived from soy and because soy consumption would not adversely affect nutritional status (*22*). Subjects consumed daily one main soy dish (½ cup of soybeans or 38 g of texturized vegetable protein) and two soy snacks—either soy chips (a roasted soybean product) or a spread for crackers made from the whole soybean. The estimated isoflavone content was about 200 mg/day, the equivalent of about 0.3 mg/day of conjugated steroidal estrogen, assuming that the estrogenic activity of phytoestrogens is about 0.1% that of conjugated estrogen.

Baird said preliminary findings indicate that, compared with control subjects, significantly more women fed soy exhibited an estrogenic response, as demonstrated by an increase in the number of superficial cells of the vaginal epithelium. She remarked that postmenopausal women were chosen for this study because of the decision to examine the estrogenic rather than the antiestrogenic effects of plant phytoestrogens. In premenopausal women with relatively high estrogen levels, the antiestrogenic effects of soybeans may have been observed.

Protease Inhibitors

Ann Kennedy, David Brandon, and Irvin Liener focused their attention on the soybean protease inhibitors. Kennedy reviewed her work, as well as that of others, in the field of protease inhibitors and cancer prevention. She noted that the soybean-derived Bowman-Birk protease inhibitor (BBI) either inhibits or prevents development of experimentally induced colon (*23*), oral (*24*), lung (*25*), liver (*23*), and esophageal cancers (von Hofe E, Newberne P, Kennedy A: unpublished observations). Protease inhibitors, at the levels used in these studies, do not adversely affect animal growth. Kennedy noted that the anticarcinogenic effect of the BBI is thought to stem from its ability to inhibit chymotrypsin activity (*26*), but results also suggest an important role for trypsin inhibition in suppression of the promotional stage of carcinogenesis (*27*). She said in vitro work indicates that protease inhibitors prevent conversion of normal cells to the malignant state even at very late stages in carcinogenesis but that they have no effect on cancerous cells (*28*). Protease inhibitors are unique in that they cause an irreversible suppressive effect on the carcinogenic process. They have also been shown to suppress oncogene expression and to inhibit carcinogen-induced protease activity (*29*).

Kennedy said recent data suggest that the antigrowth effects of raw soybeans commonly attributed to protease inhibitors may actually be due to an unidentified factor(s) (*30*). Furthermore, in human populations consuming soybeans, the connection between pancreatic enlargement and protease inhibitors observed in animals has not been seen. In fact, incidence of pancreatic cancer is decreased in these groups (*31*). Kennedy noted that in vitro comparisons of the pure BBI with an extract of soybeans containing BBI indicate that the activity of the soybean extract could be directly attributable to BBI (*26*). However, she said an in vivo study suggests that the extract may contain an additional anticarcinogenic agent working in conjunction with the BBI (*26*). The extract contains approximately 50% protease inhibitor; the remaining content is unknown, but it may include isoflavones as well as other potential anticarcinogens. Kennedy commented that the lowest effective dietary levels of protease inhibitors used in these animal studies (0.1%) could be achieved by humans by modifying the diet to include soy products.

Brandon discussed the measurement of protease inhibitors in soybeans and soy products, noting the concern of the Agricultural Research Service of the United States Department of Agriculture (USDA) over the possible adverse effects of

protease inhibitor intake in humans, particularly in infants (*32*). Enzyme-linked immunosorbent assays (ELISA), using monoclonal antibodies, have been developed for the measurement of two different protease inhibitors found in soybeans—BBI and Kunitz trypsin inhibitor (KTI) (*33,34*). These procedures are suitable for quantifying residual protease inhibitor levels in foods. A variety of processed soy products, a series of soybean flours derived from seeds in the USDA Soybean Germplasm Collection, and the soybean isoline L81-4590 (lacking KTI) (*35*) have been analyzed. Brandon noted that an important observation from the ELISA analysis of heat-treated soy flours derived from the isoline was that KTI, not BBI, is responsible for the heat-stable activity of commercial soy flour that inhibits trypsin activity (*36,37*). The microenvironment of the soy flour appears to promote heat inactivation of BBI to a greater extent than it affects KTI. This finding contrasts with the results of work showing that BBI is relatively heat stable in the pure form (*38*). Moisture, fat content, the presence of agents that influence changes in disulfide bonds, and interactions with other constituents, such as carbohydrates, appear to influence the denaturation of inhibitors (*39*).

Brandon said analysis of infant formula has revealed that active KTI and BBI, when measured on the basis of weight per gram of protein, are reduced to about 0.1% of their levels in raw soy (*40*). An infant on a diet consisting exclusively of soy formula would consume about 10 mg of active KTI plus BBI per day. In toasted (autoclaved) soy flour, 20%-30% of the KTI activity remains, while all of the BBI is inactivated. Analysis of tofu (soybean curd) has revealed that the protease inhibitor content varied significantly among the samples, from 4 to 30 μg of BBI and from 5 to 16 μg of KTI per gram of product. The protease inhibitor content of several soy protein isolates also varied, as much as 20-fold. Not unexpectedly, there was also a wide variation in the protease inhibitor content among varieties of soybeans. Brandon suggested that food-processing strategies could be combined with genetic approaches to optimize the protease inhibitor content of soy products.

Liener reviewed research on the potential adverse effects of consuming protease inhibitors, first noting that most work has been done with small experimental animals (*41*). Consumption of raw soybeans has two major effects: growth inhibition and pancreatic enlargement. Rats consuming raw soy flour for extended periods develop adenomatous nodules involving acinar cells of the pancreas (*42*). Additionally, raw soy flour consumption potentiates the effect of pancreatic carcinogens (*43*). In a study by Liener et al (*44*), heat treatment of raw soybeans almost completely eliminated this potentiation, while the addition of protease inhibitors to the heated product restored most of the pancreatic enlargement observed with raw soy, suggesting that protease inhibitors are at least partly responsible for pancreatic enlargement.

Liener noted that the varied response to raw soy flour among species is particularly important. Rats, mice, chickens, hamsters, and young, growing guinea pigs all exhibit pancreatic enlargement in response to protease inhibitors, while dogs, pigs, calves, and monkeys do not (*45*). Growth inhibition induced by soybean products is thought to result from a deficiency of the sulfur-containing amino acids caused by the dramatic increases in fecal levels of endogenous protease enzymes, particularly trypsin and chymotrypsin, two enzymes that are rich in these amino acids (*46*).

Commenting that pancreatic enlargement apparently stems from elevated serum levels of the hormone cholecystokinin, Liener commented that pancreatic enzyme secretion is inversely related to the level of trypsin in the intestine, a process regulated by cholecystokinin. This hormone stimulates the pancreas to produce trypsinogen, but because the protease inhibitors combine with trypsin, the suppressive effect of trypsin on intestinal release of cholecystokinin is eliminated (*47*).

Liener raised the question: Can the effects of protease inhibitors in small animals be extrapolated to humans? A negative feedback system in humans has been observed (*48*). Directly supplying BBI or raw soy flour to the duodenum causes an increase in secretion of pancreatic enzymes (*48*) and in blood levels of cholecystokinin (*49*). (BBI, in contrast to KTI, survives in gastric juice.) Despite these observations, he said, it is not possible at this time to accurately assess the health consequences of consuming processed soy products.

Phytosterols and Saponins

A. Venket Rao presented evidence for reduction of colon cancer risk by phytosterols and saponins. Both substances are common constituents of plants, but the concentration in soybeans is particularly high. Phytosterols are structurally similar to the animal sterol cholesterol. They inhibit cholesterol absorption and are almost quantitatively recoverable in fecal material, indicating that very little intestinal absorption occurs (*50*). Soybeans are a major contributor of phytosterols to the diet, particularly β-sitosterol (90 mg/100 g edible portion of the soybean) (*51*). Soybean oil is potentially an important source of phytosterols, but upon refinement and hydrogenation, phytosterol levels are reduced from 315 mg to 217 mg and 132 mg, respectively, per 100 g of oil (*51*). Dietary phytosterol intake among populations differs dramatically; the typical western diet contains about 80 mg/day, while Japanese and vegetarian diets provide about 400 and 345 mg/day, respectively (*52,53*).

In addition to the phytosterols, whole soybeans contain significant amounts of saponins, about 5% of dry weight (*54*), while tofu contains approximately half that much. Saponins are amphophilic compounds having surfactant properties and, like phytosterols, bind to cholesterol and bile acids.

Rao said that while nutritional interest in both phytosterols and saponins has focused on their cholesterol-lowering properties, some data suggest that these compounds may be anticarcinogens. In rats, β-sitosterol-supplemented diets (0.2% by weight) inhibit chemically induced colon cancer (*55*), and phytosterols reduce, in a dose-dependent fashion, cholic acid-induced colon cell proliferation and mitotic activity (*56*). Diets containing phytosterols at 1% by weight are well tolerated by experimental animals (*57*). Dietary saponins from soybeans and other sources have been shown to enhance immunity (*58,59*), are cytotoxic to Sarcoma 37 cells (*60*), inhibit DNA synthesis in tumor cells (*61*), decrease the growth of human epidermoid carcinoma cells (*62*) and human cervical carcinoma cells (*63*), and inhibit Epstein-Barr virus genome expression (*64*). Saponin-sup-

plemented diets (1% by weight), as is the case for the phytosterols, normalize abnormal colonic cell proliferative activity induced by carcinogens (Rao AV: unpublished observations).

Inositol Hexaphosphate

Ernst Graf discussed the rationale for the hypothesis in which inositol-1,2,3,4,5,6-hexaphosphate (IP_6), not fiber, is postulated to be responsible for the inverse correlation between the incidence of colon cancer and the consumption of fiber-rich foods (*65*). When the IP_6 content of cereals, fruits, and vegetables is considered, the international data suggest that there is a greater negative correlation between IP_6 and colon cancer incidence than between fiber and colon cancer incidence. IP_6 is found in a variety of plant foods, particularly cereals, but soybeans are an especially rich source, containing about 1.4% on a dry-weight basis (*66*).

Graf noted that most nutritional interest thus far has focused on the inhibitory effect of IP_6 on mineral absorption. IP_6 forms tight chelates with a variety of polyvalent metals such as calcium, zinc, and iron (*66*). However, he said, the ability to bind metal ions, particularly iron, may provide the basis for the anticarcinogenic effects of this compound. Graf commented that iron may be a key factor, via the Haber-Weiss reaction, in the production of hydroxyl radicals, which are postulated to play a role in the etiology of some cancers (*67*). IP_6 has been shown to limit the oxidant reactivity of transition metals (*66*), to inhibit lipid peroxidation (*67*), and to inhibit experimentally induced colon cancer (*68-73*). It has also been suggested that IP_6, through absorption following dephosphorylation to IP_3, could be an important second messenger involved in the regulation of cell differentiation (*73*).

Phytochemical Variation

James Duke discussed phytochemical variation in soybeans. Duke started by noting that there are over 10 000 named or numbered varieties of the common soybean *Glycine max L.* In these varieties, as one might expect, lies tremendous chemical variation. The genus *Glycine* was originally applied to a distant relative, now known as *Apios americana*, which is an edible root with more protein than is found in potato (*74*).

The isoflavone content of soybeans varies tremendously according to the plant part, variety, year harvested, and geographic location (*75*). Soybean hulls contain only relatively minor amounts of isoflavones, the majority of which occur in the hypocotyl, although one common isoflavone, genistein, is found primarily in the cotyledon (*75*). Equally significant are the reported differences in isoflavone content according to the varieties of soybeans and the year harvested. One study (*75*) reported a threefold variation in total isoflavone content among four varieties of soybeans, while a 30% variation was noted in a single variety of soybeans over a 4-year period. The content of individual isoflavones varied as much as 50%. Not surprisingly, location influences isoflavone content, even within fairly close geographical areas.

Duke noted that chemical variation is not limited to the isoflavones. In some instances as much as a fivefold variation was found among different phenolic acids in soybeans, many of which have also been investigated as potential anticarcinogens.

Isoflavones in Plant Physiology

Renee Kosslak described the role of isoflavones in defense strategies utilized by plants. Plants produce a wide range of products or secondary metabolites thought to enhance their survival (*76*). The isoflavones daidzein and genistein are the major inducers of the nodulation genes in *Bradyrhizobium* bacteria, which form nodules on soybeans (*77*).

The genetic regulation of isoflavone synthesis in plants is not well understood, in part because of the limited number of appropriate mutants affecting this pathway (*78,79*). In soybeans, near-isogenic lines that differ in their root fluorescence are being examined to determine whether they are active in genetic regulation of isoflavone synthesis (*80*). (These differences in root fluorescence in soybeans were first observed in 1934.) There are five loci that affect root fluorescence (*80*), and although specific substances responsible for this property have not been identified, isoflavones are thought to be involved. Preliminary data indicate that the levels of daidzein, genistein, and coumestrol, which is also a phytoestrogen, were either reduced or absent in root extracts from three of the nonfluorescent isolines tested (Kosslak R: unpublished observations).

Kosslak suggested that if future research implicates isoflavones and/or phytoestrogens as important dietary factors in cancer prevention and if the demand for soybean specialty products materializes, it may be possible to manipulate levels of these compounds in soybeans, using root fluorescence as a marker.

Soybean Processing

Bernie Szuhaj briefly discussed soybean processing procedures (*81-83*). Solvent extraction is the primary method of producing soybean products today. Soybeans entering the plant are first cleaned, cracked, and dehulled. Then moisture is added so they can be "flaked," leaving a product that is 3% hypocotyl, 89% cotyledon, and 8% hulls. The oil is removed from the flakes by hexane, producing defatted flakes and soybean oil. From the defatted flakes come a variety of products with a protein content, on a dry-weight basis, that ranges from about 50% for soy flour and grits to about 60%-70% for protein concentrates and about 90% for protein isolates. The primary difference between soy protein concentrates and isolates is the larger percentage of carbohydrate in the soy protein concentrates. Many commercial doughnuts contain soy flour, and, in Europe and Asia, there is particular interest in the use of full-fat soy flours for baking.

Szuhaj noted that most soybean production today goes into animal feed, while the soy protein concentrates and isolates are marketed primarily for their multifunctional properties, such as emulsifying, gelling, fat-binding, texturizing, and dough forming. Soy products play a major role in the food chain. They are added to a wide variety of foods, from cereals to chili. Some

meat products, such as ground beef, contain up to 25% soy. These products have been used in the Armed Forces' canteens since 1983 and in the federal school lunch program.

Discussion

This workshop had two objectives: *1*) to evaluate the relationship between the risk of certain cancers and consumption of soybeans, products derived from soybeans, and/or specific components of soybeans and *2*) to recommend research initiatives aimed at clarifying this relationship. The consensus of the meeting was that there are sufficient data to justify studying the impact of soybean intake on cancer risk in humans.

There were three workshop recommendations. First, future dietary studies involving soybeans should be carried out using soy products rather than isolated compounds, since soybeans appear to contain several potential anticarcinogens. Additionally, because components of food interact, both negatively and positively, with each other, the potential benefit of soy products cannot be accurately predicted solely on the basis of the effects of individual soybean components. This does not, however, prohibit future use of isolated soybean components as chemopreventive agents in clinical trials. Second, standardized and improved analytical methods are needed so that the contents of all soy-based materials employed in soybean research, whether soybean fractions or soy products, can be accurately described. This methodology will allow for valid comparisons among studies. Third, basic research on the absorption, metabolism, and physiology of potential anticarcinogens in humans should be conducted. This research will likely help to determine the clinical relevancy of these compounds and to provide a basis for selecting specific soy products for use in future dietary studies.

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The Family Guide to

GREENHOUSE
PUBLICATIONS

The family guide to natural therapies

First published in Australia by
Greenhouse Publications P/L
385-387 Bridge Road
Richmond Victoria 3121 Australia

Typeset by Solo Typesetting South Australia
Printed by Impact Printing Melbourne
Designed by Tom Kuremz

National Library of Australia
Cataloguing-in-publication data:

Beckham, Nancy.
The Family Guide to Natural Therapies.

ISBN 0 86436 143 2.

1. Naturopathy. I. Title.

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Herbs used

Internally

Echinacea *Golden seal* *Raspberry leaf* *Thyme*

Douche

Equal parts *Calendula* and *Comfrey* or *Calendula* and *Golden seal* with 3 drops *Sandalwood* or *Tea-tree oil*.

Tea-tree oil suppositories are now available.

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MENOPAUSE

General

Description

The end of menstruation.

Cause

A normal occurrence, usually taking place between the age of 45–55 years, although occasionally earlier. When the ovaries stop producing ova (eggs) the menopause will be finished. Women who have partial hysterectomies in which their ovaries are not removed, will experience menospause when their ovaries stop producing. Many women go through the transition easily.

The ovaries are responsible for producing most of the oestrogen and progesterone. These hormones can be produced in other glands, such as the adrenals, which explains why some women do not experience the symptoms to the same extent. As our hormonal system works on a feedback system, if pharmaceutical therapy is used the glands will not be stimulated to function.

Herbs used

There are a number of herbs and foods which contain small amounts of plant oestrogens and progesterone, or substances which function in a similar way.

Oestrogenic plants

Alfalfa – best used as sprouts
Aniseed *Fennel* *Liquorice* – not the confectionery
Parsley – best used fresh; a handful daily
Red clover – best used as sprouts
Sage – as a cold tea; see *Hot Flushes*, see below
Soya beans – best used as sprouts. Difficult to sprout in cold weather.

Plants with progesterone precursors

Fenugreek – sprouts are more effective
Sarsaparilla *Wild yam*

Include about one cup of sprouts per day in your diet. Professional herbalists use some other herbs which are also in these categories but they are rarely available to the general public. A Chinese herb called dong quai is commonly recommended for menopausal problems and is stocked by some health food stores.

Other herbs are also useful and these will be shown with each of the symptoms.

Hot Flushes

Description

A sudden, hot feeling, usually starting in the chest and going up to the face. Sometimes the skin gets quite red. This may be followed by heavy sweating, so a change of clothes or a warm jacket should be kept handy.

Cause

The flushing is said to be the result of the effort of the controlling centre in the brain to stimulate female hormone production but as the ovaries are no longer effective, all that is achieved is an increase in circulation which manifests as heat.

Herbs especially recommended

Sage – use a cold infusion, as explained in Chapter 4, page 140.
Alfalfa and *Red clover* sprouts
Liquorice (not the confectionery) *Ginseng*

Supplements

Vitamin E

Tissue salts

Kali Sulph

Depression and Lethargy

It is commonly believed that women who lead useful, interesting lives do not apparently exhibit the same degree of symptoms. Boredom is a common cause of depression. Don't listen to people who tell you 'it's normal to be unwell at your age'.

Herbs especially recommended

Chilli – only if there is no flushing
Ginseng *Liquorice* *Oats*

All the oestrogenic and progestogenic herbs except hops.

Other

A number of community health centres run programmes for menopausal women.
Find some new interests – you probably have another thirty years to go!

Tissue salt

Nat Mur

Nervous Agitation, Anxiety

Herbs especially recommended

Hops *Linden* *Motherwort*
(Refer also to Chapter 8)

Other

Avoid the use of tranquillisers and similar drugs; there are already over 8000000 of these prescribed in Australia each year.
Learn yoga or relaxation and practise for 15–20 minutes daily. Don't drink tea, coffee, cola, and reduce alcohol to 1 glass daily or none.

Tissue salt

Kali Phos

Osteoporosis

Description

This is the most serious change associated with the menopause and the most common reason for the prescribing of Hormone Replacement Therapy. The density of the bones throughout the body starts to decline (commonly known as brittle bones) which is why elderly women often fracture easily and have difficulty healing.

Cause

As the levels of oestrogen fall, there is a corresponding loss of calcium from the bones. In severe cases, a 70 year old woman may have lost 50 per cent of her total bone mass. Excessive coffee drinking interferes with calcium absorption. Anorexic women of any age suffer bone loss and a prolonged low calorie diet can have the same effect. Excess protein also causes calcium loss.

This information relates to most of the so-called developed countries. In certain countries there is no known osteoporosis and in others there is more in males than females which indicates that the problem is not solely linked to female hormone changes.

Herbs especially recommended

All the herbs in the oestrogen list and *Horsetail*.

Diet

Calcium supplementation

There are hundreds of published scientific research papers on calcium and its relationship to osteoporosis. A number of researchers now recommend that the dietary intake of calcium for adult females should be between 1000–1500mg. Most practitioners agree that the majority of middle aged women would not eat this quantity in their normal diet, so there is a fairly clear indication that supplementation is beneficial. The form this supplementation should take is debatable. There are many tablet formulas which incorporate calcium, not all researchers agree on the most appropriate dosage and, most importantly, not all people absorb calcium to the same degree. There is also some conflict relating to the correct method of measuring bone density and what other nutrients should or should not be taken with calcium.

Some studies indicate that female vegetarians who eat dairy products and eggs have less bone mineral loss. In certain areas of the world, where protein intake is extremely low, post-menopausal osteoporosis is unknown; however, it would not be intelligent to become protein deficient in order to preserve your bones. Another report indicated that a significant percentage of osteoporotic females are intolerant to dairy products.

Because there is so much scientific and individual variation, I suggest the following:

All adult females up to 35 years of age

One multi-vitamin supplement per day after breakfast.

Herbs used

Evening primrose oil *Motherwort*

Other herbs which may be helpful are indicated in Chapter 8.

Diet

Safflower oil – 2 teaspoons per day if not taking the *Evening primrose oil*.

Small frequent meals, increase protein intake if on a low protein diet.

Supplements

Spirulina – 2 tablets three times a day between meals.

PMT-D

Description

Depression, forgetfulness, crying, confusion, insomnia.

Causes

High progesterone, low oestrogen, high androgens. Occasionally lead toxicity.

Herbs used

Oestrogenic herbs

Alfalfa *Aniseed* *Fennel* *Liquorice* *Parsley* *Red clover* *Sage* *Soya beans*

The intake of these should be high just before the start of the PMT period up to menstruation.

Other herbs helpful in depression

Ginseng *Ginger* *Oats*

From the kitchen

Rosemary

Diet

Supplements

B complex with additional B1 and B3 *Glutamine*

PMT-H

Description

Fluid retention, weight gain, sore breasts, abdominal bloating, fatigue. Possible allergies, high adrenal hormones.

Herbs used

All the oestrogenic herbs except liquorice

Herbal diuretics such as

Corn silk *Dandelion* *Horsetail*

CAUTION Do not use Ginseng.

From the kitchen

Celery juice *Cucumber juice*

Diet

Reduce salt, coffee, tea, smoking.

Supplements

B complex with additional B6 *Vitamin E*
Potassium *Celery and juniper tablets*

PMT-P

Description

Generalised aches and pains.

Causes

Possible magnesium deficiency or calcium excess.

Diet

Supplements

Magnesium

PMT – with migraine type headache

Description

A throbbing, vascular headache affecting one side of the head, often accompanied by sensitivity to light and nausea. Usually lasts all day.

Cause

Believed to relate to low oestrogen, high progesterone.
Might also be an allergy or circulatory problem.

Herbs used

Oestrogenic herbs *Feverfew*

Other

Hot foot baths

PMT – with acne

Description

Outbreak of pimples coinciding with the premenstrual time (commonly 5–12 days before periods).

(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) Publication number: 0 135 172
A2

(12) EUROPEAN PATENT APPLICATION

(21) Application number: 84109990.6

(22) Date of filing: 22.08.84

(51) Int. Cl.⁴: A 61 K 31/35
//C07D311/36

(30) Priority: 24.08.83 JP 155603/83

(43) Date of publication of application:
27.03.85 Bulletin 85/13

(84) Designated Contracting States:
BE CH DE FR GB LI NL SE

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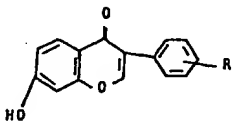
(54) Method for treatment of osteoporosis.

(57) A compound of the formula



wherein R is a hydrogen atom or a hydroxy group is effective for prevention or treatment of osteoporosis.

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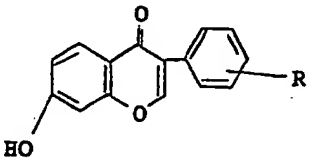
Method for treatment of osteoporosis

This invention relates to a therapeutic means for treatment of osteoporosis.

More particularly, this invention relates to a medicament for prevention or treatment of osteoporosis, which contains a compound of the formula



wherein R is a hydrogen atom or a hydroxy group.

Osteoporosis is a disease condition or illness which occurs frequently in postmenopausal females, particularly those in their sixties, and wherein the quantitative loss of bones progresses beyond a certain limit to thereby present some symptoms or risk manifestations. Among its main clinical manifestations are kyphosis, low back pain, and fractures of femoral neck, lower end of the radius, ribs, upper end of the humerus, etc. While the causative factors are variegated, including endocrine disorder and nutritional disorder, apparently the most important cause is a descreased secretion of estrogen due to hypoovarianism in females during the postmenopausal period. Therefore, of all the therapeutic agents for osteoporosis, the theoretically most effective drugs are estrogen preparations.

However, the estrogens so far available are so strong in effect as to cause side effects such as genital bleeding, mastodynia, hepatic disorder, etc. and, for this reason, have not been used recently on as many occasions as in the past. There are other types of therapeutic agents such as calcitonin, vitamin D and calcium preparations, which however are disadvantageous in that they are either only indefinitely effective or ineffective when administered by the oral route.

The present inventors have found that the compound of the formula (I) exhibits a milder estrogen activity than the conventional estrogens in the oral regimen and does not cause side effects which are produced by these known drugs but cures osteoporosis by stimulating secretion of calcitonin from the thyroid.

The compounds of the formula (I) which are employed in accordance with the present invention are invariably crystalline compounds which are white to pale yellowish brown in color, and are freely soluble in dimethylformamide and chloroform, soluble in ethanol and acetone and practically insoluble in water. When R in the formula (I) is a hydroxy group, it may be present in any position of the phenyl ring.

These compounds can be produced, for example, by cyclizing a 2,4-dihydroxy-phenyl (with or without a hydroxy group on the benzene ring) benzyl ketone to a benzopyran compound, and some of these compounds are known to have capillary vessel stabilizing activity (French Pharmaceutical Patent No. 1065), therapeutic effective for vascular disorders, inflammatory and vitamin-P deficiency disorders (United States Patent No. 3,352,754) or anticonvulsant activity (Japanese Patent Publication No. 32074/1972), but it has not been known that any of the compounds is useful for the treatment of osteoporosis.

As will be apparent from Test Example 5 which appears hereinafter, all of the compounds of the formula (I) are

sparingly toxic. Thus, in the studies in which the compounds were administered orally or subcutaneously to mice or rats at the technically feasible highest doses (5,000 to 10,000 mg/kg), there occurred no death nor toxic symptoms attributable to the compounds.

On the other hand, Test Examples 1 and 2 presented hereinafter show that 7-hydroxy-isoflavone [hereinafter referred to briefly as compound (I)] and 7,4'-dihydroxy-isoflavone [briefly, compound (II)], which are representative species of the compound represented by the formula (I), have mild estrogenic activity which is suited for the treatment of osteoporosis.

Test Example 1

Estrogenic activity of 7-hydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 6 to 7 animals. Compound (I) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally for 3 days, while as a representative example of the conventional estrogen drug, estrone was dissolved in sesame oil and administered subcutaneously for 3 days. On the fourth day, each animal was autopsied and its uterine wet weight was recorded. As shown in Table 1, compound (I) at the daily dose levels of 200 mg/kg and 400 mg/kg produced uterine weight increasing effect with a dose-response curve of moderate gradient. In contrast, estrone showed uterine weight increasing effect with a dose-response curve of steep gradient.

Table 1

Compound	Daily dose (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
Compound (I)	0 (control group)	7	35.0 ± 1.0
	6.25	7	32.8 ± 1.1
	12.5	7	33.4 ± 0.9
	25	7	35.1 ± 0.8
	50	7	35.3 ± 1.7
	100	7	35.9 ± 1.0
	200	7	57.9 ± 1.0*
	400	6	70.4 ± 6.7*
Estrone	0.0025	7	101.7 ± 4.6*
	0.005	7	159.8 ± 9.4*
	0.01	7	223.3 ± 12.5*

*: Significant as compared with control group ($P<0.01$)

Test Example 2

Estrogenic activity of 7,4'-dihydroxy-isoflavone in young oophorectomized rats

Sprague-Dawley rats, 33 days old and 11 days after oophorectomy for elimination of endogenous estrogenic activity, were used in groups of 7 animals. Compound (II) was suspended in a 1% aqueous solution of hydroxypropylcellulose and administered orally. As shown in Table 2, compound (II) at the dose level of 400 mg/kg showed mild uterine weight increasing activity.

Table 2

Daily dose of compound (II) (mg/kg)	No. of animals	Uterine wet weight (mg ± S.D.)
0 (control group)	7	31.1 ± 1.1
6.25	7	33.2 ± 0.8
25	7	32.8 ± 1.0
100	7	35.3 ± 1.3
400	7	62.3 ± 6.0*

*: Significant as compared with control group ($P<0.01$)

The following Test Examples 3 and 4 show that the compounds of this invention have bone resorption-inhibiting activity which is effective for the treatment of osteoporosis.

Test Example 3

Bone resorption inhibiting activity of 7-hydroxy-isoflavone and 7,4'-dihydroxy-isoflavone in rat fetal long bone culture.

Determination of bone resorption was performed by the method of Raisz [J. Clin. Invest. 44, 103-116 (1965)]. Thus, a Sprague-Dawley rat on the 19th day of pregnancy was subcutaneously injected with 50 μ Ci of ^{45}Ca (isotope of calcium, $CaCl_2$ solution), and was laparotomized on the following day. The embryos were aseptically taken out, the forelimbs (radius and ulna) were cut off from the trunk under a binocular dissecting microscope, and the connective tissue and cartilage were removed as much as possible to prepare bone samples. Each bone sample was preincubated at 37°C for 24 hours in 0.6 mℓ of the medium containing 2 mg/mℓ of bovine serum albumin in BGJ_b medium (Fitton-Jackson modification) [GIBCO Laboratories, Grand Island, NY 14072 U.S.A.]. Then, the sample was further incubated for 3 days in the same medium as above in which 10 μg/mℓ or 25 μg/mℓ of compound (I) or 10 μg/mℓ of compound (II) had been incorporated. Then, the radioactivity of ^{45}Ca in the medium and that of ^{45}Ca in the bone were measured and the percentage (%) of

^{45}Ca released from the bone into the medium was calculated by the following formula.

Percentage (%) of ^{45}Ca released from bone into medium

$$= \frac{\text{Count of } ^{45}Ca \text{ in medium}}{\text{Count of } ^{45}Ca \text{ in medium + Count of } ^{45}Ca \text{ in bone}} \times 100$$

As control, the bones of the embryos from the same litter were similarly incubated in the absence of compound (I) or (II) for 3 days. The mean ± standard deviation for the six bones per group are shown in Table 3. It is apparent that compounds (I) and (II) suppressed bone resorption.

Table 3

	Concentration of compound	^{45}Ca (%) released	
Control group	0	20.6 ± 3.8	19.9 ± 5.0
Test group 1	Compound (I) 10 µg/mℓ	16.5 ± 2.5*	
Test group 2	Compound (I) 25 µg/mℓ	13.5 ± 2.5*	
Test group 3	Compound (II) 10 µg/mℓ		15.9 ± 1.3**

* : A significant difference from the control group ($P<0.001$)

**: A significant difference from the control group ($P<0.002$)

Test Example 4

Inhibiting activity of 7,4'-dihydroxy-isoflavone to the bone resorption potentiating action of parathyroid hormone in rat fetal long bone culture.

The bone samples prepared in the same manner as Test Example 3 were pre-incubated for 24 hours in the same medium as that prepared in Test Example 3 which contains bovine serum album in BGJ_b medium (Fitton-Jackson modification). Then, in the concomitant presence of PTH (parathyroid hormone, a bone resorption stimulant) and compound (II), the samples

were further incubated for 3 days and the percentage of ^{45}Ca released into the medium was calculated by means of the same formula as that in Test Example 3. The results are shown in Table 4. As control experiments, the same determination was made for a control group using the medium supplemented with PTH alone. It is apparent from Table 4 that compound (II) suppressed PTH-stimulated bone resorption.

Table 4

	Concentration of compound (II)	^{45}Ca (%) released
Control group	0	30.8±4.3
Test group	10 μg/ml	23.5±3.4*

*: A significant difference from the control group ($P<0.01$)

Test Example 5

Acute toxicity

Five-week-old ICR mice and 5-week-old Sprague-Dawley rats were respectively used in groups of 10 males and 10 females, and suspensions of compound (I) or compound (II) in olive oil were administered orally [2,500, 5,000 and 10,000 mg/kg of each compound] or subcutaneously [1,250, 2,500 and 5,000 mg/kg]. The animals were kept under observation for 14 days. None of the groups showed deaths nor toxic symptoms attributable to compound (I) or (II) and, therefore, LD_{50} values could not be calculated.

The daily dosage of the compound of the formula (I) according to this invention for human beings is generally about 1 to 50 mg/kg and preferably about 5 to 20 mg/kg for oral administration, and about 200 to 600 mg can be orally taken daily, once a day or, if necessary, in 2 to 3 divided doses. The compounds are preferably formulated into such dosage forms as tablets, capsules, etc. by the established pharmaceutical procedure. Such tablets and capsules can

be prepared using suitable excipients such as lactose, starch, etc., binders such as hydroxypropylcellulose, and lubricants such as magnesium stearate. The tablets may be sugar-coated, if necessary.

The following preparation examples are given to illustrate the invention in further detail and should not be construed as limiting the scope of the invention.

Example 1 Tablets

I)	7-Hydroxy-isoflavone	200 g
II)	Lactose	15 g
III)	Starch	44 g
IV)	Carboxymethylcellulose	10 g
V)	Magnesium stearate	1 g

The above components I) through V) were admixed to prepare 1000 uncoated tablets with a diameter of 8.5 mm.

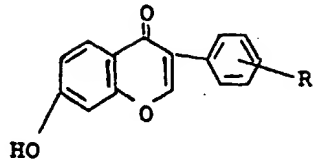
Example 2 Capsules

I)	7,4'-Dihydroxy-isoflavone	200 g
II)	Lactose	40 g
III)	Starch	50 g
IV)	Hydroxypropylcellulose	7 g
V)	Magnesium stearate	3 g

The above components I) through V) were admixed and filled into 1000 No. 1 capsules.

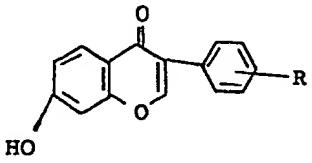
What is claimed is:

1. A compound of the formula



wherein R is a hydrogen atom or a hydroxy group for use in prevention or treatment of osteoporosis.

2. A pharmaceutical composition for prevention or treatment of osteoporosis, which contains an effective amount of a compound of the formula



wherein R is a hydrogen atom or a hydroxy group and a pharmaceutical acceptable carrier, vehicle, lubricant or diluent therefor.

3. A pharmaceutical composition according to claim 2, which is in the form of tablet, capsule, granule, fine granule, powder or syrup.

4 A pharmaceutical composition according to claim 2, wherein the osteoporosis is that caused by decreasing secretion of estrogen due to hypoovarianism.

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : A61K 31/35 — A1

(11) International Publication Number: WO 94/23716

(43) International Publication Date: 27 October 1994 (27.10.94)

(21) International Application Number: PCT/US94/04189

(22) International Filing Date: 15 April 1994 (15.04.94)

(30) Priority Data:
08/049,006 16 April 1993 (16.04.93) US

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(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

(57) Abstract

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

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METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

Summary of the Invention

The inventors have found that isoflavonoids, which are constituents of soy beans and other plants, effectively reduce the symptoms of conditions which are caused by reduced or altered levels of endogenous estrogen, e.g., menopause, and premenstrual syndrome. Without being bound by any theory, it is believed that the isoflavonoids bind to estrogen receptors, and thus exert an estrogenic response. These compounds, which are present naturally in soy-based and other plant-based foods, are safe and cause no significant side-effects. Isoflavonoids which may be administered according to the invention include genistein, daidzein, Biochanin A,

formononetin, O-desmethylangolensin, and equol; these may be administered alone or in combination.

Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

In another aspect, the invention features a therapeutic dietary product for preventing or treating symptoms resulting from reduced or altered levels of endogenous estrogen. The dietary product preferably includes a soy extract containing enriched isoflavonoids, provided in a palatable food carrier, e.g., a confectionary bar, biscuit, cereal or beverage.

Other features and advantages of the invention will be apparent from the Description of the Preferred Embodiments thereof, and from the claims.

Description of the Preferred Embodiments

Isoflavonoids are naturally occurring substances, found primarily in soy beans. These compounds are also found in lower concentrations in many other plants. Isoflavonoids can thus be administered to a patient by placing the patient on a diet containing high levels of soy-based food products, e.g., tofu, miso, soybeans, aburage, atuage and koridofu, or other plant products rich in isoflavonoids.

These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. Use of an isoflavonoid in the preparation of a medicament for preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen.

2. The use of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The use of claim 1 wherein said isoflavonoid is in a unit dosage of at least 30 mg.

4. The use of claim 1 wherein genistein and daidzein isoflavonoids are present in said medicament.

5. The use of claim 4 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

6. The use of claim 1 wherein said medicament is in the form of a dietary product.

7. The use of claim 6 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

8. The use of claim 6 wherein said dietary product is a confectionery bar containing said isoflavonoid.

9. The use of claim 6 wherein said dietary product is a cereal containing said isoflavonoid.

10. The method of claim 6 wherein said dietary product is a biscuit containing said isoflavonoid.

11. The method of claim 6 wherein said dietary product is a beverage containing said isoflavonoid.

12. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

13. The dietary product of claim 12 comprising genistein and daidzein isoflavonoids.

14. The dietary product of claim 12 wherein the food carrier is a confectionery bar.

15. The dietary product of claim 12 wherein the food carrier is a cereal.

16. The dietary product of claim 12 wherein the food carrier is a biscuit.

17. The dietary product of claim 12 wherein the food carrier is a beverage.

18. The dietary product of claim 12 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

19. The dietary product of claim 18 comprising at least about 30 mg isoflavonoids per serving.

20. The dietary product of claim 13 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/04189

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : A61K 31/35-
US CL :514/456, 899

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/456, 899

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS AND CAS ONLINE: ISOFLAVIN?, PMS, ESTRO?, PREMENSTRUAL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 3,864,362 (FEUER ET AL.) 04 FEBRUARY 1975, COLUMN 1, LINE 33 - COLUMN 2, LINE 44.	1-20 ------ 1-20

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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Date of the actual completion of the international search

24 MAY 1994

Date of mailing of the international search report

JUL 20 1994

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Form PCT/ISA/210 (second sheet)(July 1992)*

PCT/US 94/04189

6

BAR CODE LABEL

U.S. PATENT APPLICATION

08/214,194.9

SERIAL NUMBER	FILING DATE	CLASS	GROUP ART UNIT
08/049,006	04/16/93	514	1205

APPLICANT

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STATE OR COUNTRY	SHEETS DRAWING	TOTAL CLAIMS	INDEPENDENT CLAIMS	FILING FEE RECEIVED	ATTORNEY DOCKET NO.
MA	0	22	2	$442.00	05495/003001

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TITLE

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

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080 AT 05/03/93 08049006 1 201 377.00 CK

PTO-1556
(5/87)

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

APPLICANT: SHERWOOD L. GORBACH, BARRY R. GOLDIN AND HERMANN ADELCREUTZ

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METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Background of the Invention

The present invention relates to therapies for the prevention and treatment of menopausal and premenstrual symptoms.

It has long been recognized that the sharp reduction in endogenous estrogen levels which occurs prior to menopause causes a variety of unpleasant symptoms, e.g., hot flashes, nausea, nervousness, and malaise. Currently, the symptoms of menopause are treated by estrogen replacement therapy, which has recently been shown to increase the risk of certain types of cancer, such as endometrial cancer and breast cancer. Changes in levels of endogenous estrogen may also be responsible for "premenstrual syndrome", a condition occuring in younger women prior to menstruation. Premenstrual symptoms are treated with a variety of hormonal and nonhormonal therapies, which may cause side effects. Safer and more effective therapies for both conditions continue to be sought.

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Accordingly, in one aspect, the invention features a method of preventing or treating the symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of at least one isoflavonoid. The isoflavonoid may be administered in any suitable form, e.g., in the form of a plant extract rich in isoflavonoids or in the form of a purified or synthesized isoflavonoid.

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These products may not be readily available in all geographic regions (most of these foods are served predominantly in Japan), and are not be palatable to many women, particularly those accustomed to Western-style food.

Accordingly, an isoflavonoid-containing fraction can be extracted from a soy or plant product. It is preferred

that the isoflavonoids be extracted and concentrated from soy bean or soy powder. Isoflavonoids are also available commercially in substantially pure form. The concentrated isoflavonoid is preferably included in a food carrier to form a dietary product. Any type of palatable carrier may be used, but, as the isoflavonoid concentrate has a strong flavor, it is preferred that the carrier include suitable flavorings to impart a different, more palatable flavor. The dietary product may be any type of food product, e.g., a confectionary bar, biscuit, cereal or beverage.

It is preferred that the dietary product contain at least 30 mg/serving total isoflavonoids. The isoflavonoid concentrate included in the dietary product preferably includes a blend primarily comprised of genistein and daidzein. The concentrate typically also contains lower levels of other isoflavonoids. Most preferably, the dietary product contains from about 10 to 30 mg/serving, more preferably about 20 mg/serving of genistein, and from about 5 to 10 mg/serving, more preferably about 7 mg/serving of daidzein. Preferably, a dietary product containing the preferred dosage of isoflavonoids would be consumed at least once per day, preferably 1 to 2 times per day depending upon the severity of the woman's symptoms.

While it is preferred that the isoflavonoid be administered in the form of a dietary product, if desired the isoflavonoid could be administered, preferably in similar dosages, in medicament form, e.g., mixed with a pharmaceutically acceptable carrier to form a tablet, powder or syrup.

Example

The connection between diet and estrogen excretion was studied in Japanese women and men, and in a few

children. The women's mean age was 50.4 (SD 18.0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. Isoflavonoid excretion in the urine was measured in a group of three men, three women, and three children living in Kyoto and consuming the traditional diet. We found a very high excretion of isoflavonoids in the urine of these subjects. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15.5 umol per 24h in a man) and two other isoflavonoids, daidzein and equol (Table 1). All these compounds bind to estrogen receptors and have weak estrogenic activity. The excretion of the isoflavonoids in urine of the Japanese women was much higher than previously determined levels in American and Finnish women (Table 1). Excretion was high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than the levels of endogenous estrogens excreted by normal omnivorous women consuming a western or oriental diet (Table 1).

The excretion of the isoflavonoids in urine was associated with intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

It is known that Japanese women have a lower incidence of menopausal symptoms and premenstrual symptoms than the American and Finnish women.

Other embodiments are within the claims.

Table 1

Urinary isoflavonoid or estrogen (nmol/day)	Japanese/ Oriental	American	Finnish
Genistein	3440(n=3)	. .	32.1(n=12)
Daidzein	2600(n=10)	216(n=21)	40.5(n=12)
Equol	2600(n=10)	62.8(n=21)	44.2(n=12)
Oestrone (postmenstrual)	4.48(n=9)	. .	4.48(n=10)
Oestradiol (postmenstrual)	0.76(n=9)	. .	0.94(n=10)
Oestriol (postmenstrual)	4.48(n=9)	. .	4.44(n=10)

CLAIMS

1. A method of preventing or treating a medical condition in a woman caused by reduced or altered levels of endogenous estrogen, said method comprising administering to the woman an effective amount of an isoflavonoid.

2. The method of claim 1, wherein said isoflavonoid is selected from the group consisting of genistein, daidzein, Biochanin A, formononetin, O-desmethylangolensin and equol.

3. The method of claim 1 wherein said isoflavonoid is administered in a dosage of at least 30 mg.

4. The method of claim 3 wherein said isoflavonoid is administered in said dosage at least once per day.

5. The method of claim 1 wherein genistein and daidzein isoflavonoids are coadministered.

6. The method of claim 5 wherein said isoflavonoid comprises from about 10 to 30 mg genistein and from about 5 to 10 mg daidzein.

7. The method of claim 1 wherein said isoflavonoid is administered in the form of a dietary product.

8. The method of claim 7 wherein said dietary product contains at least 30 mg/serving of said isoflavonoid.

9. The method of claim 7 wherein said dietary product is a confectionery bar containing said isoflavonoid.

10. The method of claim 7 wherein said dietary product is a cereal containing said isoflavonoid.

11. The method of claim 7 wherein said dietary product is a biscuit containing said isoflavonoid.

12. The method of claim 7 wherein said dietary product is a beverage containing said isoflavonoid.

13. The method of claim 7 wherein said dietary product is consumed by said woman at least once per day.

14. A dietary product for preventing or treating symptoms of menopause, premenstrual syndrome, or conditions resulting from reduced or altered levels of endogenous estrogen, comprising at least one isoflavonoid provided in a non-soy-based palatable food carrier.

15. The dietary product of claim 14 comprising genistein and daidzein isoflavonoids.

16. The dietary product of claim 14 wherein the food carrier is a confectionery bar.

17. The dietary product of claim 14 wherein the food carrier is a cereal.

18. The dietary product of claim 14 wherein the food carrier is a biscuit.

19. The dietary product of claim 14 wherein the food carrier is a beverage.

20. The dietary product of claim 14 wherein the food carrier contains an amount of the isoflavonoid which is effective in reducing the symptoms.

21. The dietary product of claim 20 comprising at least about 30 mg isoflavonoids per serving.

22. The dietary product of claim 15 wherein said dietary product comprises from about 10 to 30 mg/serving genistein and from about 5 to 10 mg/serving daidzein.

METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

Abstract of the Disclosure

A method is provided for preventing or treating symptoms of menopause, premenstrual syndrome, or a condition resulting from reduced levels of endogenous estrogen, by administering to the woman an effective amount of an isoflavonoid. The invention also features a therapeutic dietary product, containing isoflavonoids, for preventing or treating symptoms of conditions resulting from reduced or altered levels of endogenous estrogen.

30222

#3

PATENT
ATTORNEY DOCKET NO: 05495/003001

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS, the specification of which

is attached hereto.

☒ was filed on APRIL 16, 1993 as Application Serial No. 08/049,006
and was amended on _____.

☐ was described and claimed in PCT International Application No.
filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: William E. Booth, Reg. No. 28,933; Barry E. Bretschneider, Reg. No. 28,055; Paul T. Clark, Reg. No. 30,162; Willis M. Ertman, Reg. No. 18,658; David L. Feigenbaum, Reg. No. 30,378; John W. Freeman, Reg. No. 29,066; Timothy A. French, Reg. No. 30,175; Alan H. Gordon, Reg. No. 26,168; Gilbert H. Hennessey, Reg. No. 25,759; Charles Hieken, Reg. No. 18,411; Robert E. Hillman, Reg. No. 22,837; G. Roger Lee, Reg. No. 28,963; Steven E. Lipman, Reg. No. 30,011; Gregory A. Madera, Reg. No. 28,878; Ralph A. Mittelberger, Reg. No. 33,195; Ronald E. Myrick, Reg No. 26,315; Frank P. Porcelli, Reg. No. 27,374; Eric L. Prahl, Reg. No. 32,590; Alan D. Rosenthal, Reg. No. 27,833; John M. Skenyon, Reg. No. 27,468; Michael O. Sutton, Reg. No. 26,675; Rene D. Tegtmeyer, Reg. No. 33,567; John N. Williams, Reg. No. 18,948; Gary A. Walpert, Reg. No. 26,098; Charles C. Winchester, Reg. No. 21,040; and Celia H. Ketley, Reg. No. 33,524.

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

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ATTORNEY DOCKET NO. 05495

Applicant or Patentee: SHERWOOD L. GORBACH ET AL.
Serial or Patent No.: 08/049,006
Filed or Issued: APRIL 16, 1993
For: METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: TUFTS UNIVERSITY SCHOOL OF MEDICINE
Address of Organization: BOSTON, MA
Type of Organization: UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

[X] UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
[ ] TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3))
[ ] NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)
[ ] WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE (26 USC 501(a) and 501(c)(3)) IF LOCATED IN THE UNITED STATES OF AMERICA
[ ] WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE:)
(CITATION OF STATUTE:)

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1. for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled METHOD FOR TREATMENT OF MENOPAUSAL AND PREMENSTRUAL SYMPTOMS by inventor(s) SHERWOOD L. GORBACH, BARRY R. GOLDIN, and HERMAN ADLERCREUTZ described in

[ ] the specification filed herewith.
[X] application serial no. 08/049,006, filed APRIL 16, 1993.
[ ] patent no. , issued .

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

Full Name: _____

Address: _____

[ ] INDIVIDUAL [ ] SMALL BUSINESS CONCERN [ ] NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

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Signature: _____ Date: 1/5/93

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(Accepted 24 August 1990)

Catheterisation: your urethra in their hands

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Br Med J 1990;301:905

The emphasis in undergraduate medical education is often on the theoretical aspects of medicine rather than the practical aspects. Practical procedures are commonly taught informally, the teaching being passed from one junior to the next.[1] The philosophy is of "See one, do one, teach one." Urethral catheterisation is a procedure that requires a certain amount of skill, knowledge, and experience and is not without complication,[2-4] yet it is usually delegated to the most junior and inexperienced medical staff, the junior house officers.

Subjects, methods, and results

To assess the level of competence at catheterisation among junior medical staff house officers at this hospital were interviewed with a structured questionnaire, covering three aspects of the procedure: the degree of undergraduate and postgraduate instruction, the practical and theoretical aspects of catheterisation, and, finally, problems and complications encountered.

Thirty junior house officers (graduates of five medical schools) were interviewed. Eighteen were male and 12 were female. The replies to the questionnaire showed that none of the interviewees had received any formal instruction regarding any aspect of urethral catheterisation as an undergraduate. Practical postgraduate instruction in 24 was limited to supervision of a single catheterisation, and four subjects were unsupervised. Although those interviewed had performed a mean of 28 (range 6-100) catheterisations in male patients, only four of them had catheterised female patients.

Despite the large number of procedures performed there was appreciable ignorance of the practical and theoretical aspects of catheterisation. Twenty five interviewees were unaware of the availability of short term and long term catheters or of the duration for which they may be safely left without being changed. Three interviewees simply used the catheter that was provided by the nursing staff, and one did not know that different sizes existed.

Twenty eight interviewees initially used force when meeting resistance to the passage of the catheter, and 13 stated that the development of fresh urethral bleeding would not deter them from a further attempt at catheterisation. Eighteen were happy to attempt catheterisation in a patient who had a known urethral stricture. Five interviewees were unaware of the difference between a phimosis and paraphimosis.

Despite the lack of formal tuition all had developed what seemed to be a satisfactory aseptic technique. None, however, was aware of the nature of the antiseptic fluid or the strength of the local anaesthetic gel, but simply used what was provided by the nursing staff.

Nineteen of the interviewees had encountered bleeding and six had had patients in whom a paraphimosis had developed after catheterisation. A particularly disturbing finding was that, although 14 interviewees had requested help from senior medical staff, seven were reluctant to seek advice, because of their impression that difficulties with catheterisation were not worthy of disturbing senior staff. Eight of the 12 female medical staff had encountered problems with male patients becoming sexually excited during the procedure.

Discussion

The results of our survey suggest that the technique of urethral catheterisation is poorly taught, and in the light of these results we are preparing a short teaching video to be shown to every house officer at the start of their preregistration post.

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(Accepted 8 August 1990)

Oestrogenic effects of plant foods in postmenopausal women

Gisela Wilcox, Mark L Wahlqvist, Henry G Burger, Gabriele Medley

Crops grown as animal pasture are known to have oestrogenic activity,[1] and some foods contain potential oestrogenic analogues such as the isoflavonoids (isoflavones and coumestans), lignans, and resorcyclic acid lactones,[2] which may be activated or inactivated.[3] We studied the effect of three foods reported to induce vaginal oestrus in laboratory animals[4] in postmenopausal women not taking oestrogen replacement therapy.

Br Med J 1990;301:905-6

Subjects, methods, and results

We studied 25 postmenopausal women who were non-smokers, in good general health, and taking no drugs known to affect oestrogen state (mean age 59 (range 51-70); body mass index 24·4 (range 18·7-31·6) kg/m^2; years after menopause 8·1 (range 1-20)). The protocol was a latin square design with a two week run in period and a six week experimental period. The women recorded their normal diet for 14 days and were asked to repeat the fortnightly diet throughout the study. During the experimental period the diet was

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supplemented with soya flour (45 g daily), red clover sprouts (10 g dry seed daily), and linseed (25 g daily), each for two weeks in turn. To check compliance the women returned residual food. Blood samples were taken weekly and lateral wall vaginal smears taken fortnightly and at follow up two and eight weeks after supplementation finished. Analysis was on intention to treat, but 23 women completed the study.

We examined the dependent variables vaginal cell maturation and serum concentrations of luteinising hormone and follicle stimulating hormone. The cumulative effects of the three foods at six weeks were compared with baseline by the paired *t* test, as were the residual effects, two and eight weeks after the last food supplement. We found significant differences in vaginal cytology after six weeks' supplementation ($p<0.01$, 95% confidence interval 6·0 to 17·6), which persisted for two weeks after treatment ($p<0.02$), but cytology returned to baseline after eight weeks (table).

Mean (SE) values for oestrogenic indicators in postmenopausal women consuming phyto-oestrogens

Week		Maturation value	Luteinising hormone (IU/l)	Follicle stimulating hormone (IU/l)
1			45·7 (3·1)	58·7 (2·9)
2		30·8 (4·5)	46·6 (3·4)	58·7 (3·0)
3			50·8 (8·5)	57·4 (2·9)
4		35·0 (5·1)	46·0 (3·6)	57·3 (2·9)
5	Food		46·2 (3·3)	57·7 (3·0)
6	supplementation	39·6 (5·3)	42·9 (3·2)	54·3 (2·9)
7			43·6 (3·3)	56·4 (2·8)
8		43·4 (3·6)	44·6 (3·3)	56·6 (2·4)
9			44·9 (3·5)	57·9 (2·8)
10		43·6 (4·7)	44·9 (3·3)	57·5 (2·7)
16		33·7 (5·5)		

The maturation value significantly increased after soya flour ($p<0.05$) and linseed ($p<0.02$) but not after red clover sprouts ($p=0.11$).

All women had concentrations of follicle stimulating hormone and luteinising hormone greater than those in the premenopausal range of 2-8 IU/l and 6-13 IU/l respectively. There was a cumulative effect on serum concentrations of follicle stimulating hormone ($p<0.05$) but not on luteinising hormone over the six week supplementation period. Individual two week food supplements had no measurable effects on either hormone.

In seven women with the most pronounced changes in vaginal cytology we measured serum oestradiol concentrations weekly. Baseline concentrations were <70 pmol/l in all but one woman, who was retained as the study was based on intention to treat. There were no appreciable changes in body weight during the study.

Comment

We aimed to consider whether phyto-oestrogens were of consequence in human nutrition. Our study gives some indication of the recovery time from any possible effect of treatment and also provides further evidence of causality. Vaginal maturation is a sensitive and specific indicator of oestrogenicity. Follicle stimulating hormone is less sensitive to weak oestrogenic compounds such as phyto-oestrogens. Weak oestrogenic compounds may sometimes act as anti-oestrogens, which may affect their usefulness as sources of oestrogenic activity. Conversely, tamoxifen, an antioestrogen, can have oestrogenic effects on vaginal cytology.[5]

Patterns of food intake may modulate the severity of the menopause as it is an oestrogen deficiency state. Up to half of the diet of some populations may comprise foods containing phyto-oestrogens, whereas in our study such foods comprised only about 10% of energy intake for a fairly short time. Whether menopausal symptoms differ in such populations would be worth investigation.

We thank our statistical adviser, Steve Farrish, from the department of social and preventive medicine, Monash University.

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(Accepted 19 June 1990)

Inadvertent duplicate publication

Loop diathermy excision of the cervical transformation zone in patients with abnormal cervical smears

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The *BMJ* regrets that much of the material in the above article (30 June 1990, p 1690) was substantially the same as that published previously in *Contemporary Reviews in Obstetrics and Gynaecology* (Redman CWE, Buxton EJ, Cullimore J, Luesley DM. Loop diathermy excision of the cervical transformation zone in the management of cervical intraepithelial neoplasia. 1990;2:53-8). The authors did not tell us this when the article was submitted, their article did not contain any reference to the earlier paper, and all authors signed our copyright form, which states, among other things, that "papers are accepted on condition that they have not been published by any other journal."

We regret this inadvertent duplicate publication, for which the authors hold sole responsibility, and which is in violation of our Instructions to Authors and internationally agreed guidelines.

Correction

Incidence of peptic ulcer disease in Gothenburg, 1985

An editorial error occurred in this paper by Dr Ivi-Mai Schön and others (1989;299:1132). The y axis of figure 1 should read 0, 5, 10, 15, and 20 and not 0, 0·5, 1·0, 1·5, and 2·0 as published.

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　平1−258669

⑤Int.Cl.⁴	識別記号	庁内整理番号	㊸公開　平成1年(1989)10月16日
C 07 D 311/40		7375−4C	
311/36		7375−4C	
C 07 H 17/07		7417−4C	審査請求　未請求　請求項の数 1　(全4頁)

㊹発明の名称　イソフラボン化合物の製造法

㉑特　願　昭63−83185
㉒出　願　昭63(1988)4月6日

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明細書

1．発明の名称

イソフラボン化合物の製造法

2．特許請求の範囲

大豆抽出液あるいは大豆磨砕物からイソフラボン化合物を製造するに際し、大豆中のβ−グルコシダーゼ活性が最大となるように、浸漬工程、磨砕工程あるいは磨砕後の酵素反応工程のいずれか、あるいは2以上の工程において、大豆あるいは大豆磨砕物を45〜55℃に加熱することを特徴とするイソフラボン化合物の製造法。

3．発明の詳細な説明

＜産業上の利用分野＞

本発明は大豆からイソフラボン化合物、特にそのアグリコン類を多量に含むイソフラボン化合物の製造法に関するものである。

＜従来の技術及び問題点＞

大豆にはダイジン、グリシチン、ゲニスチン、ダイゼイン、ゲニステイン等のイソフラボン化合物が含まれており、その生理活性作用はエストロゲン作用、抗酸化、抗脂血作用、抗菌作用、抗脂血、抗コレステロール作用が知られており、また最近ではガン細胞の分化誘導作用、ガン遺伝子阻害作用等、制ガン効果も確認され、その有用性が注目されている。

これらイソフラボン化合物のうち制ガン作用等の医薬的な効果は配糖体ではなく、ダイゼイン、ゲニステイン等のアグリコンが主となっている。

大豆の抽出液からイソフラボン化合物を得る方法として、例えば特開昭62−126186号公報が挙げられているが、大豆中では95％以上が配糖体として存在している為、この方法によって得られるイソフラボン化合物は配糖体が主体となり、アグリコンは極めて少量しか得ることはできない。

＜問題点を解決するための手段＞

本発明者等はイソフラボン化合物のうちでも有用性が極めて高いアグリコンを安価かつ大量に得る方法について検討したところ、大豆のイソフラボンは大豆中のβ−グルコシダーゼの作用により

容易に糖結合を切断し、そのアグリコンへ変換すること、その変換は50 ℃、pH 6.3 で最大となるという知見を得た。

本発明はこの様な知見に基づき成されたものであって、大豆抽出液あるいは大豆磨砕物からイソフラボン化合物を製造するに際し、大豆中のβ－グルコシダーゼ活性が最大となるように、浸漬工程、磨砕工程あるいは磨砕後の酵素反応工程のいずれか、あるいは2以上の工程において、大豆あるいは大豆磨砕物を45 ～55 ℃に加熱することを特徴とするイソフラボン化合物の製造法である。

以下、本発明を具体的に説明する。

原料となる大豆は酵素の失活していないものならば、どの様なものでもよく、例えば低変性脱脂大豆粉、ひき割り大豆、脱皮大豆、丸大豆等を用いることができる。

これらの大豆の抽出液からイソフラボン化合物を製造するには、大豆を5 ～10 倍の45 ～55 ℃の温水に浸漬し、その浸漬水を抽出液として精製原料とするが、浸漬水はダイゼインの比率が大豆中よりも10 %程度多くなるので、好適な精製原料と成り得る。

また大豆の磨砕物からイソフラボン化合物を製造する場合には、上記の様に浸漬した大豆を浸漬水と共に磨砕するか、あるいは常温水で浸漬して、その浸漬大豆を45 ～55 ℃で磨砕するか、あるいは磨砕後45 ～55 ℃に加熱して酵素反応させればよいが、好適には浸漬を45 ～55 ℃で行ない、これを浸漬水と共に45 ～55 ℃で磨砕し、得られた磨砕物を45 ～55 ℃に数時間保持する。こうすることにより大豆中のアグリコンの比率が大きく上昇する。

こうして得られた大豆抽出液あるいは大豆磨砕物を精製原料として用いるが、イソフラボン化合物の精製は2通りの方法がある。

1つは溶媒による精製で精製用原料、即ち大豆浸漬水あるいは大豆磨砕物を熱風乾燥、凍結乾燥等により粉末化し、これをn－ヘキサンあるいは石油エーテルで脱脂し、その残渣を乾燥後エチルエーテルで抽出してイソフラボンアグリコンのみを得る方法である。

もう一方は精製用原料の粉末化物を含水アルコールでイソフラボン化合物を環流抽出し、その抽出液を常法により濃縮、乾固させ、それを少量の含水アルコールに溶解し、これを逆相系の樹脂、例えばYMC－GEL　ODS －A　タイプ60－01（㈱山村化学研究所製）やダイヤイオンHP －20（三菱化成工業㈱製）などに吸着させた後、十分水洗し、20 %程度の含水アルコールでフェノール酸を溶出させ、次いで80 %含水アルコールでアグリコンを豊富に含むイソフラボン化合物区分を得る方法である。又、水洗の後、40 %程度の含水アルコールで配糖体画分を流出させれば、80 %含水アルコールの溶出により、アグリコン区分のみを得ることもできる。

尚、ここで用いられる逆相系の樹脂は有機溶媒、例えばアルコール系、アセトン系等で洗浄再生が容易であり、繰り返し利用できる。

又、精製用原料を粉末化しないで直接樹脂に吸着させて精製することも可能であり、浸漬水の場合には、そのまま、又磨砕物の場合には常法により濾過した濾液あるいは遠心分離した上澄液を逆相系の樹脂に接触させ、以下上記と同様に精製する。

以下、実験例を示し本発明の効果を説明する。

実験例1

脱皮大豆を5 倍量の20 ～80 ℃の水に6 時間浸漬し、その浸漬水と浸漬大豆を直ちに冷却、凍結させる。それを凍結乾燥機にて乾燥、粉末化し、その一定量を80 %メタノールで環流抽出し、定容したものの一定量を高速液体クロマトグラフィー（Waters 社209 D 型）にて分析した。

結果を第1表に示す。

第　1　表

浸漬温度	アグリコン率（%）			
	浸漬大豆 + 浸漬水		浸漬水	
	ゲニステイン*	ダイゼイン**	ゲニステイン*	ダイゼイン**
20 (℃)	3	5	−	−
30	8	8	−	−
40	14	16	20	36
45	18	24	23	39
50	23	29	24	40
55	21	26	33	45
60	12	15	18	36
70	6	7	−	−
80	4	4	−	−

* $\frac{\text{ゲニステイン(mg)}}{\text{ゲニステイン(mg)+ゲニステン(mg)}} \times 100$

** $\frac{\text{ダイゼイン(mg)}}{\text{ダイゼイン(mg)+ダイジン(mg)}} \times 100$

実験例1、2の結果から明らかな様に浸漬温度45～55℃で、又浸漬水のpH 6.3でイソフラボンアグリコンへの変換が最大となる。

＜実施例＞

以下に実施例を示す。

実施例1

脱皮大豆を5 kgを50℃の温水25 ℓ中に浸漬し、50℃で保温しながら2時間浸漬した。

次いで、その浸漬水をエバパレーターにて濃縮、乾固し、精製原料450 gを得た。これをソックスレー抽出器を使い、n－ヘキサンにて脱脂した。次いで、その残渣を十分乾燥した後、エチルエーテルにて抽出し、イソフラボンのアグリコン0.5 gを得た。

実施例2

実施例1と同様に浸漬した脱皮大豆を浸漬水と共に50℃にて磨砕し、その磨砕物を50℃、1時間保持したものを凍結乾燥機にて乾燥、粉末化し、精製原料4.1 kgを得た。これを実施例1と同様に、n－ヘキサンによる脱脂、エチルエーテルによるアグリコンの抽出を行ない、イソフラボンアグリコン7.2 gを得た。50℃の磨砕及びその温度での1時間保持によりアグリコン率は60 %以上になった。

実験例2

脱皮大豆をpH 5.5～11に調整した5倍量の50℃の温水に2時間浸漬し、その浸漬水をpHを酸性にして後、直ちに0.45 μmのフィルターにて濾過後、実験例1と同様に高速液体クロマトグラフィーにて分析した。

結果を第2表に示す。

第　2　表

浸漬水pH	アグリコン率（%）	
	ゲニステイン	ダイゼイン
5.5	33	42
6.3	39	43
6.9	35	39
8.0	33	35
9.0	19	23
10.0	17	20
11.0	15	16

実施例3

低変性脱脂大豆（日清ソーヤフラワー）10 kgに50℃の水、50 ℓを加え、1時間撹拌した。これをスプレードライにて熱風乾燥し、精製原料を得た。精製原料に対し5倍量の80 %熱メタノールによりイソフラボン類を抽出し、減圧乾固して粗イソフラボン画分103 gを得た。これを少量のメタノールに再溶解し、充填剤としてODS－Aタイプ60－01（㈱山村化学研究所製）をつめたφ70 mm×100 cmのカラムに通して吸着させた。

次いで、40 %のメタノールでフェノール酸やイソフラボン配糖体画分を流出させ、除去し、次いで80 %メタノールで溶出し、これを減圧乾固したところ、アグリコン9.5 gを得た。

実施例4

脱皮大豆を実施例1.と同様の方法で浸漬処理し

て得た没流水25 ℓに、合成吸着剤ダイヤイオンHP－20（三菱化成工業㈱製）1 kgを加え、1時間攪拌しながら、イソフラボン化合物を吸着させた。次いで、その樹脂を濾別して、20 %のエタノールで洗浄してフェノール酸を除去し、次いで80 %エタノールで溶出させイソフラボン化合物を得た。これの減圧乾燥後の重量は1.1 gであった。このイソフラボン化合物中には約40 %のアグリコンが含有していた。

特許出願人　キッコーマン株式会社

Japanese Patent Laid-Open No. 1-258669

Description

1. Title of the invention

Process for production of isoflavone compounds

2. Claims

A process for production of isoflavone compound from soybean extract or soybean mash comprising heating the soybean or soybean mash to 45 - 55°C in any of soaking process, grinding process or enzymatic reaction process after grinding, or in two or more processes thereof in order to maximize β-glucosidase activity in the soybean.

3. Detailed description of the invention

〈Field of the Invention〉

The present invention relates to a process for production of an isoflavone compound, especially an isoflavone compound containing large quantity of aglycones.

〈Prior art and problems〉

Soybean contains isoflavone compound such as daidzin, glycitin, genistin, daidzein, genistein, etc. The physiological action thereof has been known in estrogenic action, antioxidant action, antihemolytic action, antimicrobial action, antilipidemic action and anticholesterol action. Recently, anticancer action such as the differentiation inducing action

for cancer cells, inhibitory action against oncogene, etc. have confirmed, and the usefulness thereof is given attention.

In the isoflavone compounds, pharmaceutical effect such as anticancer action, etc. is found not in the glycoside but mainly in the aglycon such as daidzein, genistein, etc.

A method for obtaining isoflavone compounds from soybean extract is mentioned, for example, in Japanese Patent Laid-Open No. 62-126186. Since 95% or more of isoflavones in soybeans are found in the form of glycoside, the isoflavone compound obtained by that method is found mainly as the glycoside, consequently only small quantity of aglycon can be obtained.

〈Means for solving problems〉

The inventors of the present invention have studied a method for obtaining aglycon having extremely high usefulness among isoflavone compounds, and have obtained a knowledge that the glycoside linkage of the soybean isoflavone was easily cleaved by an action of β-glucosidase in the soybean to convert into its aglycon and the conversion reached maximum at 50°C and at pH 6.3.

The present invention was completed based on such the knowledge. Accordingly, an aspect of the present invention includes a process for production of isoflavone compound from soybean extract or soybean mash comprising heating the soybean or soybean mash to 45 - 55°C in any of soaking process, grinding process or enzymatic reaction process after grinding, or in two or more processes thereof in order to maximize β-glucosidase activity in the soybean.

The present invention will be explained in detail as follows.

Raw material soybeans may be of any types of soybeans, for example low denatured defatted soybean powder, cracked soybeans, husked soybeans, round soybeans, etc., as long as soybean enzymes are not inactivated.

For production of the isoflavone compound from soybean extract, soybeans are soaked in five to ten times volume of warm water at 45 - 55°C, and the soak water is used as the extract for purification. However the soak water contains about ten percents higher quantity of daidzein than soybeans, consequently it can be used as a preferable raw material for purification.

When the isoflavone compound is produced from the soybean mash, soaked soybeans as mentioned above are mashed with soak water, or soybeans are soaked in water at ordinary temperature and the soaked soybeans are mashed at 45 - 55°C, or after mashing soaked soybeans, the mashed soybeans are treated enzymatically under heating at 45 - 55°C. Preferably, the soaking is performed at 45 - 55°C and the soaked soybeans are mashed together with soak water at 45 - 55°C, and the obtained mashed soybeans are maintained at 45 - 55°C for several hours. By such treatment, ratio of aglycon in the soybean is largely increased.

The thus obtained soybean extract or soybean mash are used as a raw material for purification, and two types of method can be applied for purification of the isoflavone compound.

The one method is purification by using solvent wherein the raw material for purification, i.e. the soybean soak water or the soybean mash, is subjected to hot-air drying or freeze drying to produce powder, and the powder is defatted with n-hexane

or petroleum ether, then the residue is dried and extracted with ethyl ether to obtain isoflavone aglycon alone.

Another method is as follows. Powdered raw material for purification is refluxed with aqueous alcohol to extract isoflavone compounds, and the extract is concentrated and dried in the usual way, dissolved in a small amount of aqueous alcohol, adsorbed onto reverse phase resin such as YMC-GEL ODS-A type 60-01 (YMC Co.) and Diaion HP-20 (Mitsubishi Chemical Corp.), fully washed with water, and treated with about 20% aqueous alcohol to elute phenolic acid, and subsequently treated with 80% aqueous alcohol to obtain isoflavone compound fraction containing abundantly the aglycon. Further, after washing with water, only the aglycon fraction can be obtained by the procedure of elution with 80% aqueous alcohol after removing glycoside fraction by eluting with about 40% aqueous alcohol.

The reverse phase resin used herein can be easily regenerated by washing with an organic solvent such as alcohol, acetone, etc. and can be used repeatedly.

Further, the raw material for purification can be purified by adsorbing directly to the resin without making powder. When the soybean soak water is used, it can be contacted directly with the reverse phase resin, or when the mashed soybean is used, a filtrate prepared by conventional filtration or a supernatant prepared by centrifugation is contacted with the reverse phase resin, then purification is performed by the same way as mentioned above.

Effect of the present invention will be explained hereinbelow with reference to Experiment examples.

Experiment example 1

Husked soybeans were soaked with 5 times volume of water at 20 - 80°C for 6 hours. The soak water and the soaked soybeans were immediately cooled and frozen. The frozen material was freeze dried by using freeze dehydration equipment and then powdered. A certain amount thereof was refluxed and extracted with 80% methanol to prepare constant volume of the extract. A certain amount of the extract was analyzed by high performance liquid chromatography (Waters Corp. Type 209 D).

Results are shown in Table 1.

Table 1

Soaking temperature	Ratio of aglycon (%)			
	Soaked soybeans + Soak water		Soak water	
	Genistein*	Daidzein**	Genistein*	Daidzein**
20(°C)	3	5	-	-
30	8	8	-	-
40	14	16	20	36
45	18	24	23	39
50	23	29	24	40
55	21	26	33	45
60	12	15	18	36
70	6	7	-	-
80	4	4	-	-

$$* \quad \frac{\text{Genistein (mg)}}{\text{Genistein (mg) + Genistin (mg)}} \times 100$$

$$** \quad \frac{\text{Daidzein (mg)}}{\text{Daidzein (mg) + Daidzin (mg)}} \times 100$$

Experiment example 2

Husked soybeans were soaked with five times volume of warm water adjusted to pH 5.5 - 11 at 50°C for 2 hours. The soak water was adjusted to acidic pH, and immediately filtered through 0.45 μm filter, then was analyzed by high performance liquid chromatography in the same way as in example 1.

Results are shown in Table 2.

Table 2

Soak water pH	Ratio of aglycon (%)	
	Genistein	Daidzein
5.5	33	42
6.3	39	43
6.9	35	39
8.0	33	35
9.0	19	23
10.0	17	20
11.0	15	16

As can be seen from the results of Experiment examples 1 and 2, the conversion to isoflavone aglycon is maximized at the soaking temperature of 45 - 55°C, or pH of the soaking water at pH 6.3.

〈Examples〉

Examples are shown hereinbelow.

Example 1

Five kg of husked soybeans were soaked in 25 lit. of warm water at 50°C and were soaked for 2 hours while maintaining at 50°C.

Subsequently, the soak water was concentrated by using evaporator and dried to obtain 450 g of the raw material for

purification. The material was defatted with n-hexane by using Soxhlet extractor. After drying completely the residue, the residue was extracted with ethyl ether to obtain 0.5 g of isoflavone aglycon.

Example 2

Husked soybeans soaked in the same way as in example 1 were mashed together with the soak water at 50°C, and the mashed material was maintained at 50°C for 1 hour, dried and pulverized by using freeze dehydration equipment to obtain 4.1 kg of the raw material for purification. The material was defatted with n-hexane and extracted the aglycon with ethyl ether in the same way as in example 1 to obtain 7.2 g of isoflavone aglycon. Mashing at 50°C and maintaining for 1 hour at the same temperature resulted to exhibit 60% or more of aglycon content.

Example 3

To 10 kg of the low denatured defatted soybean (Nisshin soya flour) was added 50 lit. of water at 50°C, and stirred for 1 hour. The mixture was subjected to hot-air drying by using a spray-dryer to obtain the raw material for purification. Isoflavone was extracted by using 80% hot methanol, a five times volume to the raw material for purification, and dried in vacuo to obtain 103 g of a crude isoflavone fraction. This was dissolved again in small amount of methanol and passed through a column (ϕ70 mm × 100 cm) packed with ODS-A Type 60-01 (YMC Co.) to adsorb the material thereon.

Subsequently, phenolic acid fractions and isoflavone glycoside fractions were eluted and removed with 40% methanol,

then further eluted with 80% methanol and the eluate was dried in vacuo to obtain 9.5 g of aglycon.

Example 4

To 25 lit. of a soak water obtained by subjecting the husked soybeans to soaking treatment in the same way as in example 1 was added 1 kg of a synthetic adsorption resin, Diaion HP-20 (Mitsubishi Chemical Corp.) and an isoflavone compound was made to adsorb with stirring for 1 hour. Subsequently, the resin was filtered and separated, then the resin was washed with 20% ethanol to remove phenolic acid, and eluted with 80% ethanol to obtain the isoflavone compound. Weight of the compound after drying in vacuo was 1.1 g. The aglycon was observed to contain about 40% in the isoflavone compound.

⑲日本国特許庁(JP)　⑪特許出願公開

⑫公開特許公報(A)　昭61-246124

⑤1Int.Cl.⁴	識別記号	庁内整理番号	㊸公開　昭和61年(1986)11月1日
A 61 K 31/35 // C 07 D 311/30	ADU	6640-4C	審査請求　未請求　発明の数 1　(全7頁)

㊹発明の名称　制癌剤

㉑特　願　昭60-89770
㉒出　願　昭60(1985)4月24日

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明　細　書

1. 発明の名称

制癌剤

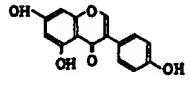
2. 特許請求の範囲

5,7,4'-トリヒドロキシイソフラボン(ゲニステイン)を有効成分とする制癌剤

3. 発明の詳細な説明

(産業上の利用分野)

本発明は　式



で示される 5,7,4'-トリヒドロキシイソフラボン(一般名 ゲニステイン)を有効成分とする制癌剤に関する。

(従来の技術)

ゲニステインは，ジャーナル・オブ・ザ・ケミカル・ソサエティー(Journal of the Chemical Society) 3447頁 1951年に記載されている公知化合物である。同文献によれば，ゲニステインはある種のクローバー(Trifolium subterraneum L.)から単離された化合物で，弱いエストロジェン作用を有することが報告されている。しかし，制癌作用については全く報告されていない。

(発明の作用および効果)

本発明者等は，土壌より分離されたシュードモナス属に属する微生物の発酵生産物中に制癌作用を有する物質を認め，さらに探索した結果，この物質がゲニステインであることをつきとめ発明を完成した。

以下，本発明の化合物の制癌作用および毒性等を説明する。

① 腫瘍細胞増殖阻止作用及びDNA合成阻止作用

ゲニステインの制癌作用を，以下の実験的腫瘍細胞の増殖阻止及びDNA合成阻止試験により調べた。

(イ) ラウス肉腫ウイルスによるラット形質転換細胞(RSV-3Y1細胞)に対する増殖阻止

試験

(ロ) ヒト上皮性癌細胞（A431細胞）に対する増殖阻止試験

(ハ) SV40ウイルスによるラット形質転換細胞（SV 40－3Y1細胞）に対する増殖阻止試験

(ニ) マウス肥満細胞腫（P815細胞）に対するDNA合成阻止試験

(ホ) マウス胸腺（EL－4細胞）に対するDNA合成阻止試験

試験方法および結果

上記(イ)，(ロ)および(ハ)の試験方法は以下の通りである。

(イ) RSV－3Y1細胞，(ロ) A431細胞または(ハ) SV40－3Y1細胞を2%牛胎児血清（ギブコ（Gibco）社製）及び各種濃度のゲニステインを含むダルベッコー（Dulbecco）のMEM（日本水産㈱製）培地中で培養した。ゲニステインの濃度は無添加，1 μg/ml，3 μg/mlおよび10 μg/mlの4通りとした。1，2，3および4日後に5% CO_2 培養器で24時間培養後，[^{3}H]チミジン（Thymidine）（アマシャム・ジャパン㈱製）を0.1 μCi/ウエル添加し，更に18時間培養した。ウエルごとに細胞をグラスファイバーフィルター（ワットマン（Whatman）GF/C）上に取り，フィルターは乾燥後シンチレーションバイアルに入れ，トルエンシンチレーターを加え，液体シンチレーションカウンターで[^{3}H]チミジン（Thymidine）の取り込みを測定した。結果を第2図に示す。

第2図に見られるように，培養液中に，3μg/mlのゲニステインが存在するとP815細胞では約50%チミジンの取り込みが抑えられ，10μg/ml濃度ではP815細胞，EL－4細胞ともにチミジンの取り込みが完全に阻止される。

② チロシン特異的リン酸化酵素活性の阻止作用

ゲニステインの各種酵素活性阻止作用を，以下の3種（a～c）のチロシン特異的プロテインキナーゼ，2種（d，e）のセリン，スレオニンプロテインキナーゼ，及びその他の酵素（f～h）について測定した。

(a) ラウス肉腫ウイルス由来（Src遺伝子 pp60^{src}）チロシン特異的リン酸化酵素

(b) ヒト上皮性癌細胞増殖因子受容体（EGFレセプター，A431細胞）チロシン特異的リン酸化酵素

(c) ネコ肉腫ウイルス由来（fes遺伝子，pp110^{fes}）チロシン特異的リン酸化酵素

(d) c－AMP依存性プロテインキナーゼ

(e) ホスホリラーゼキナーゼ

(f) ホスホジエステラーゼ

(g) Na^+，K^+－ATPase

(h) 5′－ヌクレオチダーゼ

この中，(a)～(c)は癌遺伝子由来のチロシン特異的リン酸化酵素であり，(d)(e)はセリン，スレオニンのプロテインキナーゼである。

ゲニステインによるこれらの酵素活性阻止作用の測定方法および結果を次に示す。

測定方法

(a) ラウス肉腫ウイルス由来(Src遺伝子 $pp60^{src}$)チロシン特異的リン酸化酵素活性の測定法(エム，エス・コレット，アール，エル・エリクソン：プロシーディング・オブ・ザ・ナショナル・アカデミー・オブ・サイエンシズ・オブ・ザ・ユーエスエー75巻2021～2024頁 1978年参照)

ラウス肉腫ウイルス(RSV)でトランスフォームした3Y1細胞(ラット胎児腎由来線維芽細胞)を培養し，洗浄後それにRIPAバッファー[0.5%NP40, 0.1%ソディウムデオキシコレート(sodium deoxycholate), 50mMトリス－塩酸(Tris-HCl) pH 7.2, 1mMフェニルメチルスルホニルフルオライド(phenylmethyl sulfonyl fluoride)(PMSF), 0.15M NaCl]を加え，0℃30分間放置することにより可溶化する。これを10万×g 20分間遠心することにより得た上清に，RSVを接種して担癌としたウサギより得た抗血清を加え0℃で30分～1時間インキュベートし，$pp60^{src}$と抗体を反応させる。免疫複合物をプロテインA－セファローズ4B(protein A－Sepharose－4B)(ファルマシア社製)と混合することにより集めてからRIPAバッファーで洗う。得られた$pp60^{src}$－抗体－プロテインA－セファローズ4B複合体を，20mM Pipes－NaOH pH 7.2, 5mM $MgCl_2$, 1mM DTT, 10μM[γ－^{32}P]ATP(2mCi/mmol)中で30℃5分間反応してプロテインキナーゼ反応を行った後SDSを含む反応停止液を加え，3分間煮沸し反応を止める。反応液を8%SDS－ポリアクリルアミドゲルで電気泳動し，オートラジオグラフィののち，切り出した$pp60^{src}$の放射能を液体シンチレーションカウンターにより計測し，リン酸化反応を定量した。

(b) ヒト上皮性癌細胞増殖因子受容体(EGFレセプター，A431細胞)チロシン特異的リン酸化酵素活性の測定法(エス・コウエン，ジイ・カーペンター，エル・キング；ジャーナル・オブ・バイオロジカル・ケミストリー255巻，4834～4842頁1980年参照)

EGFレセプターを多量に含むことの知られているヒト上皮性癌細胞(A431細胞)より調整した細胞膜を酵素源として用いた。50μl中に，20mM Pipes－NaOH pH 7.2, 10mM $MgCl_2$, 3mM $MnCl_2$, 1mM DTT, 10μM[γ－^{32}P]ATP(2mCi/mmol), A431細胞細胞膜(タンパク量10μg)及びゲニステインを含む反応液を5分間反応したのち，反応を停止させ，反応液を8%ポリアクリルアミドゲル電気泳動－オートラジオグラフィで解析して，分子量17万のEGFレセプターのリン酸化の有無を調べる。さらにそのEGFレセプターを切り出し，液体シンチレーションカウンターで放射能を測定することにより，リン酸化の程度を定量した。

・A431細胞からの細胞膜調整法

7%牛胎児血清(ギブコ社製)を含むダルベッコーのMEM(日本水産㈱製)培地で培養したA431細胞を集め，コーエンらの方法(スタンレイ・コーエン，ヒロシ・ウシロ，クリスタ・ストシェック，ミカエル・チンカーズ：ジャーナル オブ バイオロジカル ケミストリー257巻1523－1531頁 1982年参照)により細胞膜小胞を調整した。

(c) ネコ肉腫ウイルス由来(fes遺伝子，$pp110^{fes}$)チロシン特異的リン酸化酵素活性の測定法(アール・エー・フェルドマン，ティー・ハナフサ，エッチ・ハナフサ；セル22巻757～765頁 1980年参照)

ネコ肉腫ウィルスによりトランスフォームしたラット3Y1細胞，及びこの細胞を接種して担癌としたフィッシャーラットの血清を用いて，$pp60^{src}$の場合と同様にして免疫沈降した$pp110^{fes}$のプロテインキナーゼ活性を測定した。

(d) c－AMP依存性プロテインキナーゼの活性測定法

ウサギ筋肉より調整したc－AMP依存性プロテインキナーゼ（タンパク量4μg）（シグマ（Sigma）社製）を50mM Hepes－NaOH pH 7.5, 10mM $MgCl_2$, 4μM[γ-^{32}P]ATP（2mCi/mmol）, 6mg/mlヒストン type ⅡA（シグマ社製）, 10μM c－AMP及びゲニステインを含む反応液50μl中で30℃5分間反応した。2×2cmのワットマンロ紙P81にスポットし，ロ紙を50mM NaClでで5分間ずつ4回洗浄後，さらにアセトンで5分間洗浄し，液体シンチレーションカウンターで放射能を計測した。

(e) ホスホリラーゼキナーゼ活性の測定法

50μl中に40mMトリス－塩酸（Tris－HCl）pH 7.4, 100μM $CaCl_2$, 1mM DTT, 10mM $MgCl_2$, 10μM[γ-^{32}P]ATP.（2mCi/mmol）, 10μg ホスホリラーゼb（phosphorylase－b）（シグマ社製），ウサギ筋肉ホスホリラーゼキナーゼ（phosphorylase kinase）（タンパク量2μg）（シグマ社製）及びゲニステインを含む反応液を30℃5分間反応後，SDSを含む反応停止液を加え100℃で2分間煮沸し反応をとめた。ホスホリラーゼbのリン酸化は反応液を8％SDS－ポリアクリルアミドゲル電気泳動－オートラジオグラフィ後，切り出したホスホリラーゼbを液体シンチレーションカウンターで測定することにより定量した。

(f) ホスホジエステラーゼ活性の測定

50μl中に，50mMトリス－塩酸（Tris－HCl）pH 7.5, 8mM $MgCl_2$, 0.8mM EDTA, 0.02mM DTT, 5mM c－AMP（シグマ社製），ウシ心臓ホスホジエステラーゼ（タンパク量10μg）（シグマ社製），及びゲニステインを含む反応液を37℃30分間反応する。

10％TCAを50μl加えて反応をとめ，5,000rpm 10分間遠心して得た上清90μlを用いてリンの定量を行う。リンの呈色反応は，上清液に1％トリトンX－100 5μl，精製水350μl，2.5％モリブデン酸アンモニウムを含む5N－硫酸水溶液50μlを加え20分間放置後，660nmの吸光度を測定することにより定量した。

(g) Na^+, K^+－ATPase活性の測定法

50μl中に，50mMトリス－塩酸（Tris－HCl）pH 7.5, 60mM NaCl, 25mM KCl, 2mM $MgCl_2$, 0.1mM EDTA, 3mM ATP, イヌ腎臓より調整したNa^+, K^+－ATPase（タンパク量560ng）及びゲニステインを含む反応液を37℃30分間反応後，ホスホジエステラーゼと同様にして反応の結果生じたリンを定量した。

◦ Na^+, K^+－ATPaseの調製

Na^+, K^+－ATPaseは，カワムラらの方法（カワムラ，オータ，ナガノ；ジャーナルオブバイオケミストリー87巻1327-1333頁 1980年参照）イヌ腎臓外髄（outer medulla）を50mMイミダゾールpH 7.4, 0.25Mスクロース，1mM EDTA, 0.1mM ATPを含むバッファー中でポリトロン（polytron）（キネマティカ（Kinematica）社製）で破壊後超遠心することにより得られたミクロソーム画分をSDSで抽出することにより調製した。

(h) 5′－ヌクレオチダーゼ活性の測定法

50μl中に55mMトリス－塩酸（Tris-HCl）pH 8.5, 5.5mM $MgCl_2$, 1.1mM ATP, 10mM 酒石酸ナトリウムカリウム塩，5′－ヌクレオチダーゼ（蛇毒）（シグマ社製）及びゲニステインを含む反応液を，37℃3分間反応後，ホスホジエステラーゼと同様にして反応産物のリン酸を定量した。

結　果

ゲニステインの各酵素に対する活性阻止作用

酵素系		ID_{50}（μg/ml）
(a)	$pp60^{src}$ プロテインキナーゼ	0.8
(b)	EGFレセプタープロテインキナーゼ	0.7
(c)	$pp110^{fps}$ プロテインキナーゼ	6.5

(d) c-AMP依存性プロテインキナーゼ	>100
(e) ホスホリラーゼ キナーゼ	>100
(f) ホスホジエステラーゼ	>100
(g) Na^+, K^+ - ATPase	>100
(h) 5'-ヌクレオチダーゼ	>100

ID_{50}：50%阻止量

以上の結果に示されるように，ゲニステインは癌遺伝子由来のチロシン特異的リン酸化酵素活性を特異的に阻止する。

チロシン特異的リン酸化酵素は，癌細胞の増殖に関与すると考えられているから，この酵素活性の特異的阻止作用が認められたことは，ゲニステインの制癌作用を裏付けるものである。

③ C57BL/6 系マウスを用い，ゲニステインを腹腔内に注射して急性毒性を調べた。LD_{50} は 500㎎/kg 以上であった。

上記 腫瘍細胞増殖阻止作用，DNA 阻止作用およびチロシン特異的リン酸化酵素活性の阻止作用の試験結果より，ゲニステインはすぐれた制癌作用を有しており，しかも急性毒性の結果も低いので，ヒトおよび動物の癌の治療，癌の転移に伴う疾患の治療および再発の予防のための制癌剤として有用である。

ゲニステインの臨床投与量は活性成分として，通常成人1日当り，200～1,000㎎であり，これを1～4回に分けて投与する。投与量は患者の状態や年令等，個々の場合に応じて適宜調節される。

ゲニステインは単独で治療に供されるほか，他の化学療法剤あるいは免疫療法剤と併用される。併用される化学療法剤としては，サイクロホスファミド，ビンブラスチン，ビンクリスチン，アドリアマイシン，6-メルカプトプリン，5-フルオロウラシル，マイトマイシンC，ブレオマイシン，アクラシノマイシン，ネオカルチノスタチン，シトシンアラビノシド，シスプラチン，アクチノマイシンD，ニトロソウレア系薬剤等が挙げられる。

また，免疫療法剤としては，たとえば，クレスチン，BCG，ピシバニール，レンチナン，インターフェロン，インターロイキン等が挙げられる。これらの薬剤と併用する場合の投与量はゲニステイン1に対し，併用薬剤 0.001～10 程度が適当である。

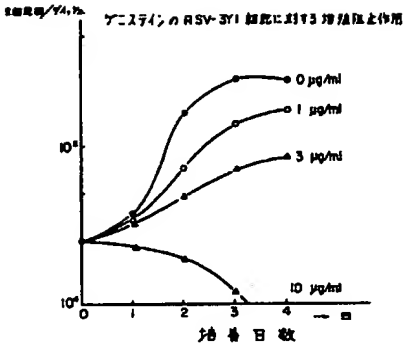
ゲニステインの投与は，経口剤（錠剤，カプセル剤，液剤）あるいは非経口剤（直腸投与製剤，注射剤，ペレット）の製剤形態で行なわれる。これ等の製剤は，任意慣用の製剤用担体あるいは賦形剤を通常の方法によって配合された組成物として調製される。この際使用される担体あるいは賦形剤は，一般的に用いられるもので良く，たとえば，錠剤の場合，水，ブドウ糖，乳糖，アラビアゴム，ゼラチン，マンニトール，でん粉ペースト，マグネシウムトリシリケート，タルク，トウモロコシでん粉，グラチン，コロイドシリカ，馬鈴薯でん粉，尿素等が利用できる。また液剤は，水性または油性の懸濁液，溶液，シロップ，エリキシル剤であってもよく，これらは通常の方法で調製される。直腸投与のためには，坐剤用組成物として提供され，基剤としては，通常用いられるもの，たとえばポリエチレングリコール，ラノリン，カカオ脂，ウイテプゾル® （ダイナミットノーベル社）等を使用できる。

4. 図面の簡単な説明

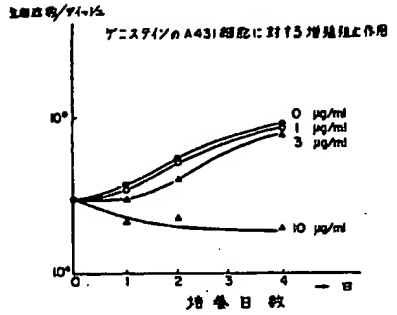
(1) 第1図(イ)，(ロ)および(ハ)はゲニステインのRSV-3Y1細胞，A431細胞およびSV40-3Y1細胞に対する増殖阻止作用を示す。

(2) 第2図はゲニステインのP815およびEL-4細胞に対するDNA合成阻止作用を示す。

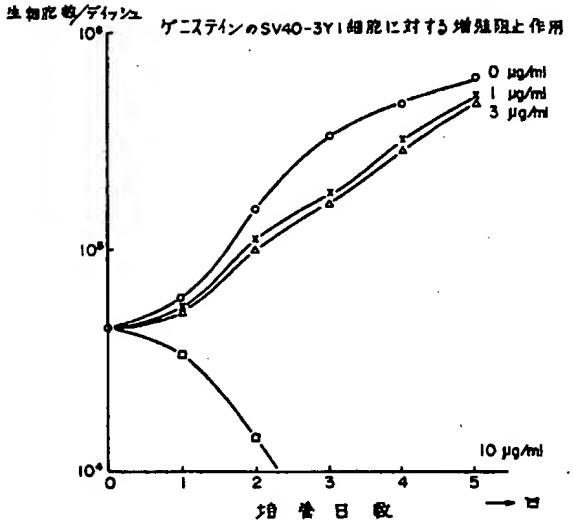
第 1 図 (イ)



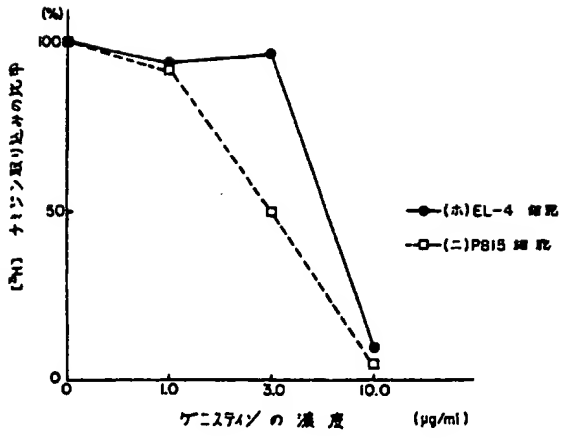
第 1 図 (ロ)



第 1 図 (ハ)



第 2 図



手 続 補 正 書 (自発)

昭和60年5月23日

特許庁長官 志 賀 学 殿

1. 事件の表示
昭和60年特許願第89770号

2. 発明の名称
制癌剤

3. 補正をする者
事件との関係 特許出願人
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5. 補正の対象
明細書の「発明の詳細な説明」の欄

6. 補正の内容
別紙の通り

特許庁 60.5.25 出願第二係

(1) 明細書第4頁第2行「(イ)～(ロ)」を「(イ)～(ヘ)」に訂正する。
(2) 明細書第6頁第3行「Ｓｒｃ」とあるを「ｓｒｃ」に訂正する。
(3) 明細書第7頁2行、「Ｓｒｃ」とあるを、「ｓｒｃ」に訂正する。
(4) 明細書第8頁第10行「反応して」を「反応させて」に訂正する。
(5) 明細書第9頁7行「調整」とあるを「調製」に訂正する。
(6) 同頁下から第1行、「調整」とあるを、「調製」に訂正する。
(7) 明細書第10頁8行、「調整」とあるを「調製」に訂正する。
(8) 明細書第11頁3行、「調整」とあるを「調製」に訂正する。
(9) 同頁第10行「反応した。」を「反応させた。」に「ロ紙」を「濾紙」に訂正する。
(10) 同頁第11行「ロ紙」を「濾紙」に訂正する。
(11) 同頁第11行「で」を削除する。
(12) 明細書第13頁10行「調整」を「調製」に訂正する。
(13) 明細書14頁11行「3分間」を「30分間」に訂正する。

(19) Japan Patent Office (JP)

(12) LAID-OPEN PATENT APPLICATION (A)

(11) No. J61-246124

A CARCINOSTATIC AGENT

(21) Filing no.: 60-89770

(22) Filing date: APRIL 24, 1985

(43) Laid-open: NOVEMBER 1, 1986.

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(74) Agent: Patent Attorney Kiyonari FUJINO and one other

Examination request: Not yet made

Number of invention: 1

(Total 7 pages)

(51) Int.Cl.[4]	Identification Code	JPO Classification
A61K 31/35	ADU	
// C07D 311/30		6640-4C

Specification

1. Title of Invention

A carcinostatic agent.

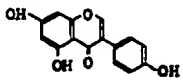
2. Patent Claims

A carcinostatic agent containing, as an effective component, 5,7,4'-trihydroxyisoflavone (genistein).

3. Detailed Description of the Invention

Technical sphere of the invention

This invention relates to a carcinostatic agent containing, as an effective component, 5,7,4'-trihydroxyisoflavone (generic name: genistein) represented by formula:



Technology of the Prior Art.

Genistein is a known compound described in the Journal of the Chemical Society, p. 3447, 1951. According to the said literature, genistein is a compound isolated from a certain species of clover (Trifolium subterraneum L.) and is reported to have a weak estrogen action. However, there have been no reports whatsoever regarding a carcinostatic action.

Action and effect of the invention.

Among the fermentation products from a microbe belonging to Pseudomonas isolated from soil, these inventors found a substance having carcinostatic action and identified that the said substance was genistein as a result of carrying out further investigations. This invention was completed on the basis of this discovery.

The carcinostatic action of the compound of this invention, toxicity or the like will now be described in greater detail.

[1] Tumour cell proliferation inhibitory action and DNA synthesis inhibitory action

The carcinostatic action of genistein was investigated with experimental tumour cells using the following proliferation inhibition tests and DNA synthesis inhibition tests.

(A) Proliferation inhibition test with respect to Rous sarcoma virus-transformed rat cells (RSV-3Y1 cells).
(B) Proliferation inhibition test with respect to human epithelial cancer cells (A431 cells).
(C) Proliferation inhibition test with respect to SV40 virus-transformed rat cells (SV40-3Y1 cells).
(D) DNA synthesis inhibition test with respect to mouse mastocytoma (P815 cells).
(E) DNA synthesis inhibition test with respect to mouse thymus (EL-4 cells).

Test methods and results.
The test methods of aforesaid (A), (B) and (C) were as follows:

(A) RSV-3Y1 cells, (B) A431 cells or (C) SV40-3Y1 cells were cultured in Dulbecco MEM media (made by Nippon Suisan KK.) containing 2 % bovine foetal serum (made by Gibco Co.) and various concentrations of genistein. Four levels of genistein concentrations were prepared, namely, 0 μg/ml, 1 μg/ml, 3 μg/ml and 10 μg/ml. At 1, 2, 3 and 4 days later, the viable cell count per dish was determined using trypan blue. The results are shown in Figure 1 (A)-(C).

As shown in Figure 1, genistein was found to have cell proliferation inhibitory action with a quantity of addition of around 1-3 μg/ml, and a marked proliferation inhibitory action was observed at 10 μg/ml.

The test methods of the aforesaid (D) and (E) were as follows:

(D) P815 cells or (E) EL-4 cells were suspended in RPMI 1640 medium (made by Nippon Suisan KK.) to which had been added 2 % bovine foetal serum inactivated at 56°C for 30 minutes (made by Flow Laboratories Co.) and 80 μg/ml gentamicin (Essex Japan KK.), and the final cell concentration was adjusted to 2×10^5 cells/ml. The said cell suspension was plated on a 96-well flat-bottomed microplate (made by Sumitomo Bakelite KK.) in an amount of 200 μl/well, and genistein was added to a final concentration comprising 0 μg/ml, 1 μg/ml, 3 μg/ml and 10 μg/ml. This plate was cultured in an incubator for 24 hours at 37°C and 5 % CO_2, and thereafter [^{3}H]-thymidine (made by Amersham Japan KK.) was added in an amount of 0.1 μCi/well, and the plate was further incubated for 18 hours. The cells on each well were separately placed on a glass fibre filter (Whatman, GF/C), and the filter dried and thereafter introduced into a scintillation vial, a toluene scintillator added, and the [^{3}H]-thymidine uptake was measured using a liquid scintillation counter. The results are shown in Figure 2.

As seen in Figure 2, when genistein 3 μg/ml was present in the culture medium, the thymidine uptake was suppressed by about 50 % for the P815 cells, and the thymidine uptake was completely inhibited at a concentration of 10 μg/ml for both the P815 cells and EL-4 cells.

[2] Inhibitory action on tyrosine-specific kinase activity

Various enzyme inhibitory actions of genistein were measured for the following three types of tyrosine-specific protein kinases (a-c), two types of serine-threonine protein kinase (d, e) and other enzymes (f-h).

(a) Rous sarcoma virus-derived (src gene $pp60^{src}$) tyrosine-specific kinase.
(b) Human epithelial tumour cell growth factor receptor (EGF receptor, A431 cells) tyrosine-specific kinase.
(c) Feline sarcoma virus derived (fes gene, $pp110^{fes}$) tyrosine-specific kinase
(d) c-AMP-dependent protein kinase
(e) Phosphorylase kinase
(f) Phosphodiesterase
(g) Na^+,K^+-ATPase
(h) 5'-nucleotidase

Among these, (a)-(c) are tyrosine-specific kinases derived from tumour genes, and (d) and (e) are serine-threonine protein kinases.

The measurement methods for the enzyme inhibitory actions of genistein and the results obtained are shown below.

Measurement methods.

(a) Measurement method of Rous sarcoma virus-derived (src gene $pp60^{src}$) tyrosine-specific kinase activity (cf. M. S. Collette, R. L. Erikson: Proceeding of the National Academy of Sciences of the USA, Vol. 75, pp. 2021-2024, 1978).

3Y1 cells (rat foetal kidney-derived fibroblast cells) transformed with Rous sarcoma virus (RSV) were cultured, washed, and thereafter thereto was added RIPA buffer [0.5 % NP40, 0.1 % sodium deoxycholate, 50 mM tris-HCl, pH 7.2, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 0.15 M NaCl], and the cells were lysed by leaving to stand at 0°C for 30 minutes. The cell lysate was centrifuged at 100,000 x g for 20 minutes, and to the thereby obtained supernatant was added

antiserum obtained from a rabbit which was made cancer bearing by inoculation of RSV. The mixture was incubated at 0°C for from 30 minutes to 1 hour, and thereby $pp60^{src}$ and the antibody were allowed to react. The immune complex was collected by mixing with protein A-Sepharose-4B (made by Pharmacia Co.), and thereafter was washed with RIPA buffer. The protein kinase reaction was carried out by allowing the obtained $pp60^{src}$-antibody-protein A-Sepharose-4B complex to react in 20 mM Pipes-NaOH pH 7.2, 5 mM $MgCl_2$, 1 mM DTT, 10 μM [γ-^{32}P]ATP (2 mCi/mmol) at 30°C for 5 minutes, thereafter a reaction stop solution containing SDS was added, and the reaction was stopped by boiling for 3 minutes. The reaction liquor was subjected to electrophoresis on 8 % SDS-polyacrylamide gel, an autoradiograph was taken, and thereafter the radioactivity of the cut out $pp60^{src}$ was measured using a liquid scintillation counter and the phosphorylation reaction was assayed.

(b) Measurement method of human epithelial tumour cell growth factor receptor (EGF receptor, A431 cells) tyrosine-specific kinase (cf. S. Coen, G. Carpenter, L. King: Journal of Biological Chemistry, Vol. 255, pp. 4834-4842, 1980).

Cell membranes prepared from human epithelial tumour cells (A431 cells) which are known to contain a large amount of EGF receptor were used as the source of enzyme. The reaction liquor, containing in 50 μl, 20 mM Pipes-NaOH pH 7.2, 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM DTT, 10 μM [γ-^{32}P]ATP (2 mCi/mmol), A431 cell membrane (protein content 10 μg) and genistein was reacted for 5 minutes, and thereafter the reaction was stopped and the reaction liquor was analysed using 8 % polyacrylamide gel electrophoresis – autoradiography, and the presence or absence of phosphorylation of the EGF receptor of molecular weight 170,000 investigated. Furthermore, the said EGF receptor was cut out, the radioactivity was measured using a liquid scintillation counter, and the degree of phosphorylation thereby assayed.

• **Cell membrane preparation method from A431 cells.**
A431 cells cultured on Dulbecco MEM medium (made by Nippon Suisan KK.) containing 7 % bovine foetal serum (made by Gibco Co.) were collected and the cell membrane vesicles were prepared in accordance with the method of Coen et al. (cf. Stanley Coen, Hiroshi Ushiro, Krista Stochek, Michael Tinkars: Journal of Biological Chemistry, Vol. 257, pp. 1523-1531, 1982).

(c) Measurement method of feline sarcoma virus derived (fes gene, $pp110^{fes}$) tyrosine-specific kinase activity (cf. R. A. Feldman, T. Hanafusa, H. Hanafusa; Cell, vol. 22, pp. 757-765, 1980).

Using rat 3Y1 cells transformed with feline sarcoma virus and also serum of Fischer rat which was made cancer bearing by inoculation of the said cells, immunoprecipitation was carried out in the same way as for $pp60^{src}$, and the activity of the thereby obtained protein kinase of $pp110^{fes}$ was measured.

(d) Measurement method of c-AMP-dependent protein kinase activity.

c-AMP-dependent protein kinase prepared from rabbit muscle (protein content 4 μg) (made by Sigma Co.) was allowed to react in 50 μl reaction liquor containing 50 mM Hepes-NaOH pH 7.5, 10 mM $MgCl_2$, 4 μM [γ-^{32}P]ATP (2 mCi/mmol), 6 mg/ml histone type IIA (made by Sigma Co.), 10 μM c-AMP and genistein, at 30°C for 5 minutes. The reaction liquor was spotted onto 2 x 2 cm Whatman filter paper P81, the filter paper was washed 4 times with 50 mM NaCl for 5 minutes each time, and furthermore was washed with acetone for 5 minutes, and the radioactivity was measured using a liquid scintillation counter.

(e) Measurement method of phosphorylase kinase activity.

The reaction liquor containing, in 50 μl, 40 mM tris-HCl, pH 7.4, 100 μM $CaCl_2$, 1 mM DTT, 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP (2 mCi/mmol), 10 μg phosphorylase-b (made by Sigma Co.), rabbit muscle phosphorylase kinase (protein content 2 μg) (made by Sigma Co.) and genistein was allowed to react at 30°C for 5 minutes, and thereafter a reaction stop solution containing SDS was added and the reaction was stopped by boiling at 100°C for 2 minutes. The phosphorylation of phosphorylase-b was measured by a method wherein the reaction liquor was subjected to 8 % SDS-polyacrylamide gel electrophoresis – autoradiography, and thereafter the cut out phosphorylase-b was measured using a liquid scintillation counter.

(f) Measurement method of phosphodiesterase activity.

A reaction liquor containing, in 50 μl, 50 mM tris-HCl, pH 7.4, 8 mM $MgCl_2$, 0.8 mM EDTA, 0.02 mM DTT, 5 mM c-AMP (made by Sigma Co.), bovine heart phosphodiesterase (protein content 10 μg) (made by Sigma Co.) and genistein was allowed to react at 37°C for 30 minutes.

The reaction was stopped by the addition of 10 % TCA 50 μl, the reaction liquid was centrifuged at 5,000 rpm for 10 minutes, and the phosphorous was assayed using the thereby obtained supernatant 90 μl. The colour reaction of phosphorus was assayed by a method wherein 1 % Triton X-100 5 μl,

purified water 350 µl and 5 N-sulphuric acid aqueous solution 50 µl containing 2.5 % ammonium molybdate were added to the supernatent, the mixture was left to stand for 20 minutes, and thereafter the absorbance was measured at 660 nm.

(g) Measurement method of Na^+,K^+-ATPase activity.

A reaction liquor containing, in 50 µl, 50 mM tris-HCl, pH 7.5, 60 mM NaCl, 25 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 3 mM ATP, Na^+,K^+-ATPase prepared from canine kidney (protein content 560 ng) and genistein was allowed to react at 37°C for 30 minutes, and thereafter the phosphorus generated as a result of the reaction was assayed in the same way as in phosphodiesterase.

• Preparation of Na^+,K^+-ATPase.

The Na^+,K^+-ATPase was prepared by the method of Kawamura et al. (cf. Kawamura, Ota, Nagano: Journal of Biochemistry, Vol. 87, pp. 1327-1333, 1980) wherein the outer medulla of canine kidney was destroyed with polytron (made by Kinematica Co.) in a buffer containing 50 mM imidazole pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.1 mM ATP, and thereafter centrifuged and the thereby obtained microsome fraction was extracted with SDS.

(h) Measurement method of 5'-nucleotidase activity.

A reaction liquor containing, in 50 µl, 55 mM tris-HCl, pH 8.5, 5.5 mM $MgCl_2$, 1.1 mM ATP, 10 mM potassium sodium tartrate, 5'-nucleotidase (snake venom) (made by Sigma Co.) and genistein was allowed to react at 37°C for 30 minutes, and thereafter the phosphoric acid in the reaction product was assayed in the same way as for the phosphodiesterase.

Results.

Activity inhibitory action of genistein with respect to various enzymes

Enzyme system	ID_{50} (µg/ml)
(a) $pp60^{src}$ protein kinase	0.8
(b) EGF receptor protein kinase	0.7
(c) $pp110^{fes}$ protein kinase	6.5
(d) c-AMP-dependent protein kinase	> 100
(e) Phosphorylase kinase	> 100
(f) Phosphodiesterase	> 100
(g) Na^+,K^+-ATPase	> 100
(h) 5'-nucleotidase	> 100

ID_{50}: 50 % inhibition quantity

As shown by the above results, genistein specifically inhibits tumour-derived tyrosine-specific kinase activity.

The tyrosine-specific kinase is considered to be involved in the proliferation of tumour cells, and therefore the fact that a specific inhibitory action was found on this enzyme activity supports the carcinostatic action of genistein.

[3] Using C57BL/6-series mice, acute toxicity was investigated by intraperitoneal injection of genistein. LD_{50} was 500 mg/kg or more.

In the light of the above test results of the tumour cell proliferation inhibitory action, DNA inhibitory action and tyrosine-specific kinase activity inhibitory action, genistein has excellent carcinostatic action, and moreover the results for the acute toxicity are shown to be low, and therefore genistein is useful as a carcinostatic agent for the therapy of human and animal tumour, the therapy of diseases accompanied by tumour metastasis and the prevention of recrudescence.

The clinical dose of genistein is usually 200 – 1,000 mg as the active component per adult per day, and this is administered once daily or divided by 2 - 4 times. The administration dose is suitably adjusted according to the individual case such as in accordance with the condition, age and the like of the patient.

Genistein may be used for therapy on its own, or can be used in combination with another chemotherapeutic agent or immunotherapeutic agent. As co-used chemotherapeutic agent, cyclophosphamide, vinblastine, vincristine, adriamycin, 6-mercaptopurine, 5-fluorouracil, mitomycin C, bleomycin, aclacinomycin, neocartinostatin, cytosine arabinoside, cisplatin, actinomycin D, nitrosourea-series agent or the like may be proposed.

Moreover, as immunotherapeutic agent, for example, krestin, BCG, picibanil, lentinan, interferon, interleukin or the like may be proposed. The dosage for combination with these agents is appropriately 0.001-10 with respect to genistein 1.

The administration of genistein may be carried out in the agent forms of an oral agent (tablet, capsule agent, liquid agent) or a parenteral agent (rectal administration agent, injection, pellet). These formulations may be prepared as compositions formulated with an arbitrary conventional carrier or excipient in accordance with the usual methods. The carrier or excipient used in such case may be a

generally used species, and for example, in the case of tablets, water, glucose, lactose, gum Arabic, gelatine, mannitol, starch paste, magnesium trisilicate, talc, corn starch, gelatine (sic), colloidal silica, potato starch, urea or the like can be used. Moreover, the liquid agent may be aqueous or an oily suspension, solution, syrup or elixir, and these may be prepared by the usual methods. For rectal administration, the agent is prepared as a composition for a suppository, and as base agent, a usually used species, for example, polyethylene glycol, lanolin, cocoa butter, Witepsol ® (Dynamit Nobel Co.) or the like can be used.

4. Brief Explanation of the Figures

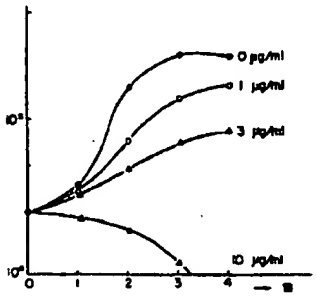
(1) Figure 1 (A), (B) and (C) show the proliferation inhibitory action of genistein with respect to RSV-3Y1 cells, A431 cells and SV40-3Y1 cells.

(2) Figure 2 shows the DNA synthesis inhibitory action of genistein with respect to P815 and EL-4 cells.

Table 1 (A)

Proliferation inhibitory action of genistein with respect to RSV-3Y1 sells

Viable count/dish

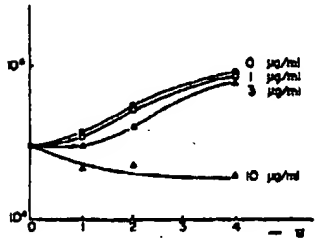


Number of days cultured

Table 1 (B)

Viable count/dish

Proliferation inhibitory action of genistein with respect to A431 sells



Number of days cultured

Table 1 (C)

Proliferation inhibitory action of genistein with respect to SV40-3Y1 sells

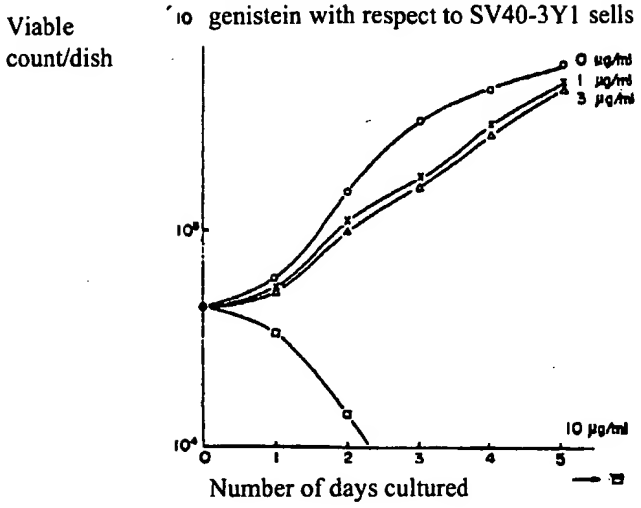
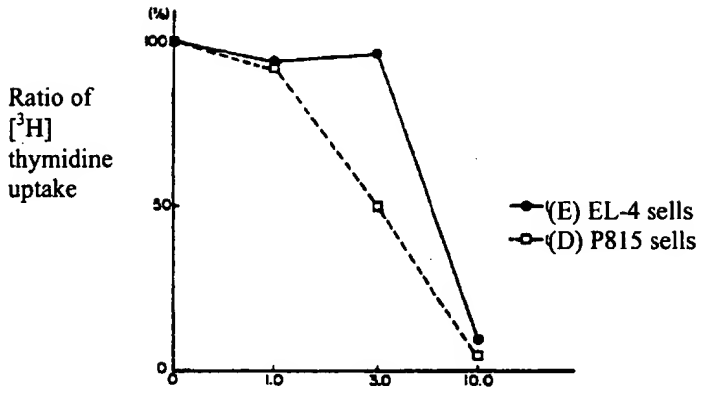


Table 2



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in newborn babies support an essential role for n-3 fatty acids in retinal development.[5]

The DHA content of erythrocytes is small compared with that of grey-matter, but the fatty acid composition of the erythrocyte membranes may indicate the fatty acid status of neural and perhaps other membranes. During the period of most rapid DHA accumulation in the developing rat, diet-induced changes in neural DHA are reflected in red blood cell DHA.[6]

Dietary n-3 fatty acids can also modify endogenous prostaglandin production and perhaps by this means influence uterine prostaglandins and gestation time. In the Faroe Islands, where birthweights are amongst the highest in the world with long gestation periods and rapid fetal growth, the intake of marine fat rich in n-3 fatty acids is high and erythrocyte DHA values in pregnant women were found to be almost twice those in normal individuals in other countries.[7]

The lower erythrocyte DHA found in patients on epoetin could be due to an increased requirement for this n-3 fatty acid as a result of increased red cell production, and this implies a deficiency of or a rate-limited production of DHA. However, plasma DHA values were not low, which raises the possibility of a defect of incorporation of this fatty acid into the membrane in patients on haemodialysis.

A low membrane DHA probably has little effect on red cell function and may be of minor importance in adults, although it is of interest that visual hallucinations have been described in patients on epoetin.[8] Unlike the adult, the fetus requires DHA in quantity for its developing nervous system, and haemodialysed patients do occasionally become pregnant. For reasons not fully understood, pregnancy in uraemia is associated with a high risk of premature labour and retarded fetal growth.[9] A lack of DHA would be detrimental to the fetus, and our results indicate that in a uraemic pregnant woman on haemodialysis, low quantities of membrane DHA could be one of the hazards to which the fetus is exposed. Because epoetin gives rise to even lower membrane DHA content, its use could increase the risk to the fetus: n-3 fatty acid dietary supplements are indicated.

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Dietary phyto-oestrogens and the menopause in Japan

SIR,—Lock, in an article on the menopause,[1] has discussed differences between Japanese women and women in western societies. Japanese women have a much lower frequency of hot flushes than women in Canada. Lock concluded that "cultural indifference to the hot flush in Japan" was unlikely to account fully for these findings.

Recently our Helsinki group studied, in collaboration with Japanese scientists, the diet and phyto-oestrogen excretion in Japanese women and men, and in a few children.[2] The women's mean age was 50·4 (SD 18·0) years and they were all from a small village south of Kyoto and consumed a traditional Japanese low-fat diet. We studied a group of three men, three women, and three children living in Kyoto and consuming the traditional diet, and in this group we measured the isoflavonoid genistein.[2] We found a very high excretion of phyto-oestrogens in urine. The mean values were almost identical in the two groups and especially high excretion was found for genistein (maximum 15·5 μmol per 24 h in a man) and two other isoflavonoids, daidzein and equol (table). All these compounds bind to oestrogen receptors and have weak oestrogenic activity.[3] The excretion of the isoflavonoids in urine of the Japanese women was much higher than in American and Finnish women (table) (ref 4 and unpublished data) and as high in children as in middle-aged and old people. These compounds were excreted in 100-fold to 1000-fold higher amounts than those of endogenous oestrogens in normal omnivorous women consuming a western or oriental diet (table).

URINARY EXCRETION OF ISOFLAVONOID PHYTO-OESTROGENS AND ENDOGENOUS OESTROGENS IN JAPANESE OR ORIENTAL WOMEN, AND IN AMERICAN AND FINNISH OMNIVOROUS WOMEN

Urinary isoflavonoid or oestrogen	Japanese/Oriental	American	Finnish
Genistein	3440 (n = 3)*	..	32·1 (n = 12)
Daidzein	2600 (n = 10)*	216 (n = 21)	40·5 (n = 12)
Equol	2600 (n = 10)*	62·8 (n = 21)	44·2 (n = 12)
Oestrone (postmenopausal)	4·48 (n = 9)†	..	4·48 (n = 10)
Oestradiol (postmenopausal)	0·76 (n = 9)†	.	0·94 (n = 10)
Oestriol (postmenopausal)	4·48 (n = 9)†	..	1·44 (n = 10)

All assays by gas chromatography/mass spectrometry in selected ion-monitoring mode with deuterated internal standards.[5,6] Women collected two to four 72 h urine samples 3–6 months apart and values are thus means of urinary excretion in individual subjects over 6–12 days. Results as geometric means in nmol/24 h.
*Values from ref 2.
†Oriental postmenopausal women (recent immigrants to Hawaii). Same women as in ref 7, but oestrogens measured by new technique.[5]

The excretion of the isoflavonoids in urine was associated with intake of soy products such as *tofu, miso, aburage, atsuage, kondofu,* soybeans, and boiled beans. All isoflavonoids are weak oestrogens and such high amounts could have biological effects, especially in postmenopausal women with low oestrogen levels. High levels of isoflavonoid phyto-oestrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women.

Supported by grants from the Medical Research Council, Academy of Finland, and S. Juselius Foundation, Helsinki.

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Nutrition/Infection Unit, Department of Community Health, Tufts University School of Medicine, Boston Massachusetts, USA — Sherwood Gorbach, Barry Goldin

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P04105 UN 6028

IN THE MATTER of European Patent Application No. 9309679.8 in the name of Graham Edmund Kelly

STATUTORY DECLARATION

I, Graham Edmund Kelly a citizen of the Commonwealth of Australia, residing at 1/47 Coolawin Road, Northbridge, New South Wales, 2063, Australia, do hereby declare as follows:

1. I am Chief Executive Office of Novogen Research Pty Ltd and I am the inventor and applicant of this application. I hold the degrees of Bachelor of Science (Veterinary) from the University of Sydney (1968); Bachelor of Veterinary Science from the University of Science (1969); and Doctor of Philosophy from the University of Sydney (1972). I have worked in the field of veterinary research, and general medical research, for approximately 25 years.

2. I have been involved in research on isoflavones and their biological effects since before May 1992.

3. I have read the Examiner's report from Examiner Thalmair dated 11 August 2003. I have additionally reviewed each of documents D2, D14, D26 and D32 cited by the Examiner.

4. I attach marked Exhibit **GK1** a copy of a declaration I made in connection with prosecution of a corresponding United States application. As set out in that declaration, the compositions/medicaments referred to in the claims of this European application have been shown in patient studies to be effective in the treatment of prostate cancer, premenstrual syndrome and menopause. These were patient studies in human subjects afflicted with the disorders of prostate cancer, premenstrual syndrome and menopause.

5. I understand the Examiner has rejected the claims insofar as they relate to use of isoflavone/phyto-estrogen extracts of soy or clover for the manufacture of a medicament relating to prostate cancer, based on documents D2 and D26.

6. In my opinion a person of ordinary skill in the area of biomedical research would understand that neither D2 nor D26 suggest the claimed use in the subject European application for the treatment of prostate cancer. D2 describes genistein as an anti-cancer drug against specific tumour cell lines in mice. The cell lines used are not specified. It is a well-recognized truth in the field of cancer research that no single anti-cancer drug has pan-anticancer activity. Indeed, there is a wide range of human cancers that are well known to be relatively insensitive to drugs that have potent anti-cancer activity against specific forms of cancers. Cancers generally recognized as having such insensitivity to anti-cancer drugs include pancreatic, renal and prostatic carcinoma. This insensitivity is reflected in the lack of effective drugs available clinically to treat such cancers. That is, it cannot be implied from any study showing a particular drug as having anti-cancer activity against a particular cancer, that the same drug will necessarily be active against any or all other forms of cancer. In my opinion, a person of ordinary skill in the art would recognise that D2 does not describe genistein as being a pan-cancer treatment. For the above reasons, I believe the person of ordinary skill in the art would consider the Examiner's position to be technically incorrect.

7. Likewise, document D26 describes extracts of soy beans giving rise to a particular "isoflavone" compound which is said to have a range of activities including estrogen action, anti-oxidation action, anti-haemolytic action, antilipemic action, cholesterol-lowering action and carcinostatic action. Compounds specifically referred to are daidzein and genistein. I do not consider that a person of ordinary skill in the art would regard D26 as teaching use of extracts of soy or clover for the manufacture of medicaments for the treatment of prostate cancer. Most of the current drugs used to treat human cancer are carcinostatic, and yet very few of them show any anti-cancer activity against human prostate cancer cells in vitro, and none of them are used clinically to treat prostate cancer because of their lack of efficacy.

8. In relation to prostate cancer, I am aware that the American Cancer Society has estimated that in 2003 there will be 220,900 new cases of prostate cancer and 28,900 people will die as a result of this cancer in the United States alone. Prostate cancer is a notoriously difficult cancer to treat. The only chemotherapeutic approach of any significance is anti-androgen therapy which is designed to block the body's production of dihydrotestosterone. Drugs with classical direct cytostatic or cytotoxic effect on cancer cells are typically not used clinically because of lack of efficacy.

9. In my opinion, a person of ordinary skill in the art would readily recognise that neither D2 nor D26 teach the invention as claimed, for example in claim 1 of the subject European application insofar as it relates to prostatic cancer.

10. I believe a person of ordinary skill in the art who had documents D2 and D26 before them, and were faced with a problem of treating prostate cancer, would not be led by these references to the invention as claimed, for example in claim 1 relating to use of an isoflavone phyto-estrogen extract of soy or clover for the manufacture of a medicament for administration in unit dosage form for the treatment of prostate cancer.

11. I understand that in relation to the claims of this European application concerned with symptoms of menopause, the Examiner regards documents D14 and D32 as particularly relevant, and considers the invention is obvious in light of these documents.

12. In my opinion a person of ordinary skill in the art would regard D14 as a speculative teaching. In this regard, my corresponding US patent application was involved in an interference in the United States Patent and Trademark Office with the patent which corresponded to D14. My application in the United States prevailed in that interference, and Dr Claude Hughes, the co-author of D32, gave a declaration in those proceedings. A copy of Dr Hughes' declaration is attached in Exhibit **GK2** and is directly relevant to the teachings in D14.

13. As set out in Dr Hughes' declaration, D14 is a speculative reference, which is indefinite, uncertain and tentative (see for example paragraphs 12, 15, and 17-21 of Dr Hughes' declaration). As set out in Dr Hughes' declaration at paragraphs 23 and 24 the language of D14 is at best tentative, and speculative.

14. D26 is a review article, and as I understand the Examiner's report, the Examiner relies on the description of D32 at page 88 right hand column, paragraph 4. I have reviewed this portion of D32 and note that it is predicated upon Chinese herbal medicine. The nature of the phyto-estrogens involved in the purported effects are not described, nor is the nature of the Chinese herbs used. It must be recognised that as review, D26 describes in the right hand column of page 88 the deleterious roles of phyto-estrogens in human disease, including vascular disease, coronary heart

disease and possible cancer initiation. On balance, the summary at the conclusion of D32 mentioned at the bottom of page 88 right hand column through page 89 is that the majority of the effects of phyto-estrogens are noxious, which directs away from using phyto-estrogens in human disease.

15. I believe that a person of ordinary skill in the art faced with documents D14 and D32, and additionally faced with a problem of treating menopause symptoms, would not consider it obvious to try extracts of soy or clover for the treatment of symptoms of menopause. In part the noxious effects of phyto-estrogen compounds would teach away from such a treatment. Further, there is nothing in D14, or D32 for that matter, to suggest that phyto-estrogen extracts of soy or clover would be useful in treating menopause symptoms.

AND I MAKE this solemn declaration by virtue of the Statutory Declarations Act 1959 as amended and subject to the penalties provided by that Act for the making of false statements in statutory declarations, conscientiously believing the statements contained in this declaration to be true and correct in every particular.

Declared at Sydney and Dated this 22 Day of October 2003

Graham Edmund Kelly

Before me: Ross Heisey

RÓSS M. HEISEY
DAVIES COLLISON CAVE
10, 10 BARRACK STREET, SYDNEY 2000
Registered Patent Attorney within the meaning of the Patents Act 1990

IN THE MATTER of European Patent Application No. 9309679.8 In the name of Graham Edmund Kelly

This is Exhibit **GK1** referred to in the Statutory Declaration of **Graham Edmund Kelly** made before me.

DATED this 22nd day of October 2003

Before me: Ross Heisey

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Registered Patent Attorney within the meaning of the Patents Act 1990

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the Application of	Examiner: J. Wilson
GRAHAM EDMUND KELLY	Group Art Unit: 1211
Application No. 08/338,567	
Filed: January 12, 1995	
For: HEALTH SUPPLEMENTS CONTAINING PHYTO-OESTROGENS ANALOGUES OR METABOLITES THEREOF	

DECLARATION OF GRAHAM EDMUND KELLY UNDER 37 C.F.R. §1.132

I, Graham Edmund Kelly, a citizen of the Commonwealth of Australia, residing at 1/47 Coolawin Road, Northbridge, New South Wales, Commonwealth of Australia, do solemnly and sincerely declare as follows:

1. I am Chief Executive Officer of Norvet Ltd. and am the inventor of the subject application.

2. I am a research scientist and hold the degrees of Bachelor of Science (Vet) from the University of Sydney (1968); Bachelor of Veterinary Science from the University of Sydney (1969); and Doctor of Philosophy from the University of Sydney (1972). I have worked in the field of medical and veterinary research for approximately twenty-five years.

3. I have read the Office Action in connection with U.S. Patent Application No. 08/338,567 by Examiner Wilson, dated 10 September 1996.

4. The health supplement composition comprising an extract from soya or clover as claimed in the patent application has been used in a series of therapeutic treatments conducted at my request and/or under my supervision. Details of these treatments are set forth below.

Compositions

Compositions comprising an extract of soya or clover were prepared in accordance with Examples 1 and 2 at pages 18 and 19 of the subject application 08/338,567. These compositions, for convenience referred to as "the inventive composition", were prepared comprising 40 mg, 80 mg, 120 mg, 160 mg and 240 mg of phyto-estrogen.

Treatments

Prostate Cancer

Two patients diagnosed with prostate cancer were treated initially with the inventive composition comprising 240 mg per day, and subsequently 120 mg per day phyto-estrogen. The PSA levels, a marker for prostate cancer, were stabilized in these patients and there has been no rise in the PSA levels subsequently. This demonstrates the treatment of prostatic cancer in these individuals.

A further patient diagnosed with malignant prostate cancer (PSA 13.1 µg/L) was treated with the inventive composition. The patient was treated with the composition comprising 160 mg per day phyto-estrogen, seven days prior to prostatectomy. Histological comparison was made of the pre-operative needle biopsy and the prostatectomy specimen. The needle biopsy revealed low grade infiltrating adenocarcinoma. The prostatectomy specimen showed mild patchy microvacuolation and prominent apoptosis (programmed cell death). Lymph nodes were negative for malignancy. The degenerative changes in the prostatectomy specimen, especially the apoptosis, show treatment of the prostatic cancer.

Benign or Cystic Breast Disease

A patient with benign or cystic breast disease was treated with 160 mg of the inventive composition administered orally on a daily basis. The patient exhibited no breast tenderness, which was maintained when the dosage level was reduced to 80 mg. Her symptoms did not return and she continues to have relief from mastalgia.

Pre-Menstrual Syndrome (PMS)

Nine women were treated with 80 mg per day of the inventive composition and were screened for the well-described symptoms of PMS including psychological, psychiatric, gynecological and personal status. Relief from PMS in these various symptoms was observed across the treatment group.

Menopause

Eight menopausal women were divided into two groups of four and treated with either 40 mg or 160 mg of the inventive composition administered orally on a daily basis. Four patients were also treated with a placebo composition. Indicators measured were incidence or severity of hot flushes, night sweats. Green score, vaginal pH, vaginal cytology and mean cholesterol levels across the treatment groups. A significant change in menstrual symptoms was observed and a dose response change was observed between the 40 mg and 160 mg dosage range. This indicating that 160 mg per day was the most effective dosage for treatment of menopausal symptoms.

5. These studies show that a composition according to the invention described and claimed in U.S. Patent Application No. 08/338,567 is effective in the treatment of:

- Prostate cancer

- Benign or cystic breast disease (mastalgia)
- Pre-menstrual syndrome
- Symptoms of menopause

6. As shown in the Examples 3 and 4 at pages 19 and 20 of the subject application 08/338,567, a composition according to the invention was effective in the treatment of elevated levels of cholesterol in the blood stream.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further, that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and such willful false statements may jeopardize the validity of the application or patent issuing thereon.

3.3.97
DATE

GRAHAM EDMUND KELLY

IN THE MATTER of European Patent Application No. 9309679.8 In the name of Graham Edmund Kelly

This is Exhibit **GK2** referred to in the Statutory Declaration of **Graham Edmund Kelly** made before me.

DATED this 22 day of October 2003

Before me: Ross Heisey

RÓSS M. HEISEY
DAVIES COLLISON CAVE
10, 10 BARRACK STREET, SYDNEY 2000
Registered Patent Attorney within the meaning of the Patents Act 1990

I, Claude L. Hughes, hereby declare the following:

1. I am a licensed physician and biomedical researcher specializing in reproductive endocrinology. I am also a consulting professor in the Department of Obstetrics and Gynecology, Duke University Medical Center, Durham, NC. As a physician, I have treated patients diagnosed with pre-menstrual syndrome or menopause.

2. I have been actively involved in biomedical research relating to the regulation and biological effects of sex hormones since the mid-1970s. I have been involved with research on isoflavones and their biological effects since before May 1992. A copy of my Curriculum Vitae is attached as Kelly Exh. 2011.

3. I have read the letter published in The Lancet by Adlercreutz *et al.*, "Dietary phyto-oestrogens and the menopause in Japan," 339:1233 (1992) (Gorbach Exh. 1004; "Adlercreutz"). In this letter, Adlercreutz cites to a earlier letter published in The Lancet by Margaret Lock, "Contested meanings of the Menopause," 337:1270-1272 (1991) (Gorbach Exh. 1010; "Lock"). On page 1271, Lock describes that peri- and post-menopausal Japanese women reported fewer episodes of hot flushes compared to peri-and post-menopausal Canadian women.

4. Lock hypothesizes that cultural differences are the reasons why there were fewer hot flushes reported by Japanese women compared to Canadian women. (*Id.*, page 1272, first column.)

5. Lock does not provide any data pertaining to differences in diet and isoflavone consumption and excretion between the Canadian and Japanese women participating in the study.

6. Adlercreutz presents a possible alternative explanation for Lock's observation that Japanese women report fewer episodes of hot flushes than Canadian women. Adlercreutz

hypothesizes that "[h]igh levels of isoflavonoid phyto-estrogens may party explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women." (Gorbach Exh. 1004, second column.)

7. Adlercreutz reports data on genistein, daidzein, and equol excretion in urine for four different groups of women: (1) three Japanese women living in Kyoto (for whom no daidzein or equol data are provided); (2) ten Japanese women from a small village south of Kyoto (for whom no genistein data are provided); (3) twelve Finnish women; and (4) twenty-one American women (for whom no genistein data are provided).

8. Footnote 2 in Adlercreutz cites to another article by Adlercreutz *et al.* published in 1991 in the American Journal of Clinical Nutrition (Gorbach Exh. 1012; "Adlercreutz II".) Adlercreutz II provides more detailed information about the Japanese women reported on in Adlercreutz.

9. According to Adlercreutz II, the mean age of the ten women recruited in the small rural village south of Kyoto was 46.8 ± 11.5 years at the time of the study and not 50.4 ± 18.0 years as reported in Adlercreutz. The latter mean and standard deviation represents the age of the men studied in Adlercreutz II. This is evident from the paragraph bridging pages 1093 and 1094 of Adlercreutz II.

10. Adlercreutz II also provides the ages of the three women in the study whom resided in Kyoto. The respective ages of these women at the time of the study was 33, 42, and 30 years This is shown on page 1099, in Table 1A. According to Adlercreutz II, these three women excreted 4.48, 3.55, and 1.85 micromoles of genistein per day, respectively. From these data, the arithmetic mean excretion of genistein is 3,293 nanomoles/day.

11. In my experience, given their ages it is highly unlikely that any of these three women were experiencing symptoms arising from menopause at the time they participated in the study described in Adlercreutz II.

12. Like Adlercreutz, Adlercreutz II reports that the ten women residing south of Kyoto excreted, on average, 2,600 nanomoles of daidzein and 2,600 nanomoles of equol per day. Since the correct age for the group of ten women is 46.8 ±11.5 years, whether this represents a mean or median value, the vast majority of these ten women would also have been pre-menopausal. Again, it is highly unlikely that more than one or two of these women were post-menopausal, and, therefore, potentially experiencing symptoms arising from menopause at the time they participated in the study described in Adlercreutz II.

13. Adlercreutz reported that these Japanese women excreted phyto-estrogens in their urine in amounts that were much higher than observed for the American and Finnish women. Adlercreutz attributes this greater excretion to the intake of soy products such as tofu, miso, aburage, atuage, koridofu, soybeans, and boiled beans.

14. The soybeans and soy-based products listed in Adlercreutz are foods, which as Adlercreutz states, are part of the traditional Japanese low fat diet. Adlercreutz states that the Japanese women in his study consumed a traditional Japanese low fat diet.

15. Adlercreutz does not provide any data on symptoms associated with menopause for any of the Japanese, American, or Finnish women whose urine was assayed for excreted isoflavones. Adlercreutz II does not provide this information either. Without this information, it is impossible to determine if the Japanese women in the study actually experienced fewer symptoms of menopause compared to the American and Finnish women in the study.

16. At the end of the Lancet letter, Adlercreutz sets out an alternative hypothesis to that advanced by Lock where it is stated that "[h]igh levels of isoflavonoid phyto-estrogens may partly explain why hot flushes and other menopausal symptoms are so infrequent in Japanese women."

17. One of ordinary skill in the art reading Adlercreutz would not conclude that the authors had established a cause and effect relationship between a traditional Japanese low fat diet and the reduced incidence of reported menopausal symptoms in Japanese women as described by Lock. There are at least two problems in Adlercreutz that prevent one from reaching such a conclusion. First, as mentioned, Adlercreutz does not report on the menopausal symptoms of the women enrolled in his study. Given the ages of the Japanese women studied, it is apparent that the majority of them were not old enough to be experiencing symptoms of menopause. Thus, a person reading Adlercreutz cannot determine whether there is in fact a difference between the Japanese and western women with respect to the incidence of their symptoms of menopause.

18. Second, even if one assumed that there were differences in the incidence of menopausal symptoms in the Japanese and western women, nothing in Adlercreutz permits one to conclude that ingesting large amounts of genistein, daidzein and/or equol caused that effect. This is because Adlercreutz does not describe a controlled experiment. Adlercreutz does not control for other variables in addition to isoflavone intake that could contribute to a difference in menopausal symptoms.

19. A biomedical researcher would know that there are other factors that might contribute to the results reported by Lock, whose hypothesis concerning cultural difference could be correct. Examples of other factors that could play a role include components of soy other than

isoflavones, such as protease inhibitors, phytosterols, and saponins, all of which are biologically active compounds. The study participants would also differ in their intake of these compounds. Furthermore, the differences reported by Lock might be attributed to genetic differences or to environmental and social factors unrelated to diet. Because Adlercreutz does not conduct a controlled experiment, it is impossible to rule in, or rule out, any of these or other variables as contributing to or causing Japanese women to report fewer symptoms of menopause.

20. Although Adlercreutz' data and the focus of the letter relate to isoflavones, there is nothing in Adlercreutz establishing that the lower incidence of hot flushes reported by Japanese women is a consequence of their ingesting relatively high amounts of isoflavones. The only scientific conclusion that Adlercreutz supports is that Japanese women living in Japan and consuming a traditional Japanese low fat diet high in soy-based foods excrete increased amounts of genistein, daidzein, and equol in their urine compared to omnivorous American and Finnish women.

21. Besides not describing a controlled experiment, Adlercreutz does not even establish a correlation between isoflavones and reduced symptoms of menopause because it does not report on menopausal symptoms in the women studied. The fact that some isoflavones have estrogen-like activities in some tissues does not mean that consumption of these isoflavones correlates with a lower incidence of reported symptoms of menopause. One cannot determine from Adlercreutz if this association exists, nor whether such an association could be causally related, because Adlercreutz does not provide data on the reported incidence of symptoms of menopause in the women studied.

22. In my view, Adlercreutz provides nothing more than an invitation to persons interested in menopause to conduct research to determine whether or not administering

isoflavones would in fact reduce the symptoms of menopause. Adlercreutz' data do not establish that consuming high levels of isoflavones, such as found in a traditional Japanese diet, either causes, or correlates with, a reduction of menopausal symptoms.

23. The language used by Adlercreutz in defining his hypothesis is, at best, tentative. This is not strong language that that would lead one of skill in the art to conclude Adlercreutz is teaching that menopausal symptoms can be reduced by consuming isoflavones. As long as statements are scientifically plausible, speculative statements are entirely permissible in the discussion section or closing remarks within scientific papers.

24. A person familiar with reading scientific papers would expect Adlercreutz to make emphatic statements regarding urinary excretion of isoflavones in women from Japan, America, and Finland because he actually measured that parameter. On the other hand, a person familiar with reading scientific papers would not expect Adlercreutz to make emphatic statements regarding menopausal symptoms because he measured none. Consistent with that, his closing speculative sentence includes the conditional, restrictive words "may partly explain."

25. Adlercreutz does not imply that as a method of treating symptoms of menopause women should consume a phytoestrogen-containing health supplement.

26. Adlercreutz does not imply that as a method of treating symptoms of menopause women should consume extracted isoflavones.

27. Adlercreutz does not imply that as a method of treating symptoms of menopause women should consume a health supplement composition containing a therapeutically effective amount of a purified phytoestrogen.

28. Adlercreutz does not imply that as a method of treating symptoms of menopause women should consume a health supplement composition containing a therapeutically effective amount of a concentrated phytoestrogen.

29. Adlercreutz does not imply that as a method of treating symptoms of menopause women should consume an effective amount of a purified naturally occurring extracted isoflavone.

30. Adlercreutz does not imply that as a method of treating symptoms of menopause women should consume an effective amount of a concentrated naturally occurring extracted isoflavone.

31. Adlercreutz provides no information about the ratio of genistein and daidzein in the foods comprising the diet that the thirteen Japanese women consumed during the study period. From the data provided by Adlercreutz, one can calculate that the ratio of genistein to daidzein in the urine of the Japanese women is 1.3:1. However, this does not mean that the ratio of genistein to daidzein in the consumed food was also 1.3:1, or even anywhere in a range from 1:2 to 2:1. If the former were the case, then the women would be absorbing, transporting, metabolizing, and excreting genistein and daidzein at identical rates, which is possible, but not likely. One cannot extrapolate from the urine data to the ratio of these two compounds in the original foods.

32. I have reviewed Gorbach Exh. 1009, an article by Eldridge *et al.* Given that the concentration of isoflavones in soy beans varies depending on a number of factors, for example variety and geographic location of growth, as taught by Eldridge, it would be impossible to accurately estimate the concentration of isoflavones in soy beans used to make the products consumed by the Japanese women mentioned in Adlercreutz. Given the variability in isoflavone concentration in soy beans, the variability of isoflavone concentration in soy-based foods, and

the lack of information concerning exactly what each Japanese woman in the Adlercreutz consumed, it would be impossible to accurately determine the amount of phyto-estrogen they consumed on a daily basis.

33. Having reviewed Adlercreutz, I find no indication that the Japanese women in the study consumed their traditional diet for any given period of time. While it is possible that they consumed such a diet for a period of at least one month, nothing in Adlercreutz indicates that they necessarily did so.

34. Pre-menstrual syndrome, unlike menopause, is not a consequence of too little estrogen. (Kelly Exh. 2016, page 5, Fig. 5.) In estrogen replete women, the symptoms occur in the menstrual cycle in relation to high progesterone levels. However, differences in either estrogen or progesterone levels are not found when symptomatic women are compared to asymptomatic women. In my opinion, Dr. Slavin is incorrect when she states that premenstrual syndrome is another condition associated with low estrogen levels. Accordingly, I do not believe that one would understand from reading Adlercreutz that Adlercreutz is teaching, either expressly or inherently, that isoflavones could be administered to treat pre-menstrual syndrome.

35. It is well recognized that breast cancer, unlike menopause, is not a low-estrogen condition. (Kelly Exh. 2017.) In my opinion, Dr. Slavin is incorrect when she states that breast cancer was another low-estrogen condition that could be treated by administering the estrogenic isoflavone component of soy. I suspect that just the opposite is the case for treating this disease. Accordingly, I do not believe that one would understand from reading Adlercreutz that Adlercreutz is teaching, either expressly or inherently, that isoflavones could be administered to treat breast cancer.

36. I have reviewed Gorbach Exh. 1014 and its attached Exh. 1. I believe that Dr. Gorbach was correctly characterizing Adlercreutz when he wrote "[i]n my view this publication does not discuss the use of extracts of soy as a treatment or prevention of menopausal symptoms . . . ."

37. I have reviewed Gorbach Exhs. 1005-1008. These articles do not mention pre-menstrual syndrome or menopause, nor do they describe any relationship between these conditions and isoflavones.

38. Adlercreutz does not teach that administration of high levels of extracted isoflavones reduces menopausal symptoms. Adlercreutz speculates that isoflavone consumption may partly explain the results reported by Lock. Adlercreutz does not mention breast cancer. A person interested in isoflavones and menopause would not have been prompted by the fact that Adlercreutz cited some supporting references to read other unrelated references, not cited by Adlercreutz, that are directed to a different medical problem, that is, breast cancer.

39. There have been several alternative hypotheses advanced to explain the observed effect of soy consumption on the incidence of breast cancer. Adlercreutz would not have lead researchers interested in sex hormones and breast cancer to conclude that the estrogenic activity of isoflavones was the mechanism through which isoflavones reduced breast cancer incidence. Since breast cancer risk is associated with exposure to estrogens, Adlercreutz' would have lead researchers away from a conclusion that the estrogenic activity of isoflavones was the mechanism through which isoflavones reduced breast cancer incidence. A suggestion that isoflavones may partly explain the why Japanese women reported reduced incidence symptoms of menopause does not say anything about the mechanism by which isoflavones act with respect to breast cancer.

40. As I have previously stated, while menopause is a condition associated with low levels of estrogens, pre-menstrual syndrome and breast cancer are not associated with low levels of estrogens. Consequently, Gorbach's conclusion that it would have been obvious to administer high levels of isoflavones to reduce pre-menstrual syndrome is not supported by the facts.

41. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, and that such willful false statements may jeopardize the validity of any patent issuing from the '837 application.

Dated: March 4, 2001

Claude L. Hughes Jr. MD, PhD

Claude L. Hughes, Jr.